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14. ABSTRACT
Scope: Prostate Cancer (CAP) is characterized by unique prostate-associated antigens; hence, it has been considered a prime candidate for immunotherapy. Despite numerous laboratory advances, clinical outcomes have been partial and transient.
Purpose: The overall goal of the proposed studies is to optimize the effectiveness of therapeutic whole-cell CaP vaccines by taking into consideration tumor-associated hypoxia as a relevant determinant of tumor antigenicity.
Major Findings: Gene expression in hypoxically cultured cells is more akin to that in tumor cells in situ than are cells grown normoxically. Transcripts of hypoxia-associated genes HURP/DLG7, CCNBI and HMMR were associated with Gleason score and with disease prognosis suggesting their potential as CaP biomarkers with prognostic value. By 2D-gel electrophoresis, we screened patient sera and detected novel hypoxic-cell reactive autoantibodies. Three potential TAAs; two hypoxia-specific (HSP70 and hnRNP L) and one hypoxia-nonspecific (HSP60) were selected for validation. The frequency of HSP60 autoantibodies was elevated in the CaP patient plasma; however the frequency of HSP70 autoantibodies was not elevated in CaP patients relative to healthy controls.
Significance: Our data suggests that hypoxically cultured CaP cells are more akin to tumor sells in situ than are cells grown normoxically. We have identified hypoxia-reactive proteins, pathways and autoantigens with potential value as biomarkers or therapeutic targets. Introduction of pO2 as a variable can constitute a tool for the development of more effective immune therapy CaP

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Introduction

Prostate cancer (CaP) remains among the most common causes of cancer–related deaths in men. Because CaP is characterized by unique prostate–associated antigens, it has been considered among prime candidates for immunotherapy. Despite numerous laboratory advances, clinical outcomes have been partial and transient. One plausible reason for the incomplete response is that vaccine cells, prepared under standard tissue culture conditions, can drastically differ in expression of macromolecules *in situ*, and thus may immunize against a less complete antigen spectrum. The purpose of the proposed studies is to optimize the effectiveness of therapeutic whole–cell CaP vaccines by taking into consideration tumor–associated hypoxia as a relevant determinant factor of tumor antigenicity. We hypothesize that hypoxically cultured CaP cells are more similar in their antigen landscape to CaP cells *in situ* than are normoxically cultured CaP cells. The following Tasks were defined in the approved statement of work; Task 1. Identify oxygen–tension responsive genes and proteins in the cells comprising a clinical–grade CaP cellular vaccine; Task 2. Validate differentially expressed molecules in CaP in association with tissue hypoxia. Our studies demonstrate that CaP cells grown under low oxygen tension (pO_2) are more antigenically similar to cells *in situ*; this will justify the evaluation of their therapeutic value in a preclinical model.

Body

Task 1. Identify oxygen–tension responsive genes and proteins in the cells comprising a clinical–grade CaP cellular vaccine.

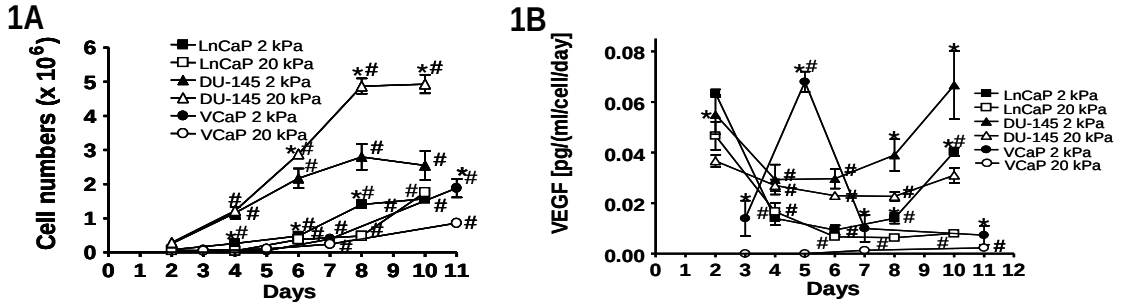
Approach: Identification of specific candidate genes with pO_2-dependent expression in CaP cells has not been established yet in the context of their antigenic relevance. In Task 1, CaP cells grown at different pO_2 were tested by state-of-the-art high throughput genomics and proteomics techniques. This approach was designed to identify pO_2–regulated tumor–associated pathways and macromolecules. To determine the antigenic potential of pO_2–regulated tumor–associated macromolecules, we tested their reactivity with the spontaneous antibodies from CaP patients and the sera of age–matched non-cancerous controls, other cancers and an autoimmune disease.

Task 1a. To propagate LnCaP and VCaP cells under pO_2–controlled conditions.

Prompted by the evidence that pO_2 modulates the biological properties of CaP tumor cells, we initiated studies aimed at using pO_2 as a tool to manipulate the antigenic signature of cells used as cellular vaccines. As a model, we used LnCaP cells, originated from a lymph node metastasis (Horoszewicz, Leong et al. 1983). These cells have been used as a component of an allogeneic whole cell vaccine tested in a phase 2 clinical trial of androgen–independent CaP (Michael, Ball et al. 2005). VCaP cells were generated from a vertebral metastatic lesion and harbor the TMPRSS2-ERG fusion (present in 40–60 percent of CaP patients) (Kumar-Sinha, Tomlins et al. 2008; Clark and Cooper 2009). These two cell lines were selected because their comparison could represent a broad spectrum of CaP patients, and may thus result in a better vaccine. For contrast we included DU-145 cells, derived from a brain metastatic tumor (Stone, Mickey et al. 1978) into initial experiments. The cells were routinely maintained in culture medium (RPMI-1640 for LnCaP and DU-145 and Dulbecco's modified MEM for DU-145 cells) supplemented with 10 percent fetal bovine serum. LnCaP cells were propagated at

different pO_2 levels in a BioSpherix chamber (Lacona, NY) with adjustable oxygen partial pressure. Humidified atmosphere was maintained at 37 °C and equilibrated with a mixture of 2% O_2 and 5% CO_2 using controlled N_2 and CO_2 gas intake. Controls were placed in a standard cell culture incubator at 37 °C in a humidified atmosphere containing 21% O_2 and 5% CO_2. Hypoxic LnCaP and VCaP cells (pO_2=2 kPa) proliferated faster than at standard cell culture conditions, however hypoxia reduced the proliferation of DU-145 cells **(Figure 1A)**. Independent of the cell proliferation rate [as observed earlier by others (Ghafar, Anastasiadis et al. 2003)], hypoxic cells secreted more VEGF **(Figure 1B)**.

Figure 1 **. Effect of hypoxia on proliferation and VEGF expression by CaP cells**



LnCaP cells (5,000 cells/cm^2), VCaP (10,000 cells/cm^2), and DU-145 (5,000 cells/cm^2), were cultured at pO_2=2 kPa or 20 kPa in T-25 flasks; Figure 1A. Live cells were counted in triplicate flasks using trypan blue exclusion to differentiate dead cells; Figure 1B. Rate of VEGF secretion in hypoxia–grown cells was higher than in normoxia–grown cells.* p<0.05 relative to pO_2=20 kPa; # p<0.05 relative to 2 days at the same pO_2 value.

Task 1b. cDNA gene microarrays and data analysis.

In response to changes in oxygen availability, cells differentially regulate a vast array of genes involved in diverse pathways such as apoptosis, metabolism or angiogenesis (Bardos and Ashcroft 2005; Bosco, Puppo et al. 2006). In preliminary experiments, we observed hypoxia-associated increase in VEGF expression by CaP and ovarian cancer cells (Knutson, G.J., Vuk-Pavlovic, S.; unpublished observations). In addition, we found numerous differences between 2-D electropherograms of lysed LnCap cells propagated at pO_2= 2 kPa and to 20 kPa. Altogether, this evidence suggests that hypoxia profoundly affects gene expression relative to normoxia. Only a handful of studies, however, have analyzed the effect of hypoxia on gene expression in CaP, especially in the context of pO_2 impact on malignant progression (Koritzinsky, Seigneuric et al. 2005; Ackerstaff, Artemov et al. 2007; Butterworth, McCarthy et al. 2008). We cultured LnCaP, VCaP, and DU-145 at pO_2= 2 kPa or 20 kPa and lysed them when data showed pO_2–related differences in VEGF secretion (4, 7 and 4 days for LnCaP, VCaP and DU-145; respectively). We isolated total RNA, verified its quality using an Agilent 2100 Bioanalyzer, and assessed the transcriptome by Affymetrix Human U133 Plus 2.0 array. Genes expressed differently between experimental groups were identified as probe sets with at least a twofold hypoxia-related expression change. We selected approximately 1450, 3700 and 1400 probe sets in DU-145, LnCaP, and VCaP cells, respectively. To relate functions, pathways, networks, and unique features to genes differently expressed

between two pO_2s, we used Ingenuity Pathway Analysis (IPA; Ingenuity Systems). Benjamini-Hochberg correction was used for multiple comparisons. Transcriptome studies revealed different gene expression in cells grown in hypoxia relative to those in normoxia. Correlation analysis between expression profiles, for all probes or hypoxia-sensitive probes revealed that LnCaP and VCaP cells were similar in gene expression changes and different to DU-145 cells (data not shown). This last finding and the similar effect of hypoxia on cell growth in LnCaP and VCaP cells could be related to the presence of androgen receptor in these cell lines and its absence in DU-145 cells (Sobel and Sadar 2005). Interestingly, regardless of cell-specific changes in gene expression profiles, hypoxia-modified genes in molecular pathways associated with cancer and urologic diseases **(Table 1)** were overexpressed in comparison to normoxic cells ($p<0.001$) in all cell lines. These data suggest an association of low pO_2 and aggressive features of CaP.

Table 1 . Transcripts for diseases and disorders preferentially affected by hypoxia

DU-145

Pathway	p-value	# molecules
Genetic Disorder	$1.11x10^{-9}$ – $5.16x10^{-3}$	558
Cancer	$2.98x10^{-9}$ – $5.50x10^{-3}$	354
Reproductive System Disease	$2.79x10^{-7}$ – $4.40x10^{-3}$	232
Developmental Disorder	$3.04x10^{-7}$ – $3.97x10^{-3}$	103
Immunological Disease	$1.00x10^{-6}$ – $5.16x10^{-3}$	253

LnCaP

Pathway	p-value	# molecules
Cancer	$2.11x10^{-7}$ – $9.17x10^{-3}$	800
Reproductive System Disease	$5.03x10^{-6}$ – $8.63x10^{-3}$	440
Genetic Disorder	$6.90x10^{-6}$ – $7.92x10^{-3}$	1393
Inflammatory Disease	$2.31x10^{-5}$ – $9.32x10^{-3}$	665
Connective Tissue Disorders	$6.42x10^{-5}$ – $9.32x10^{-3}$	433

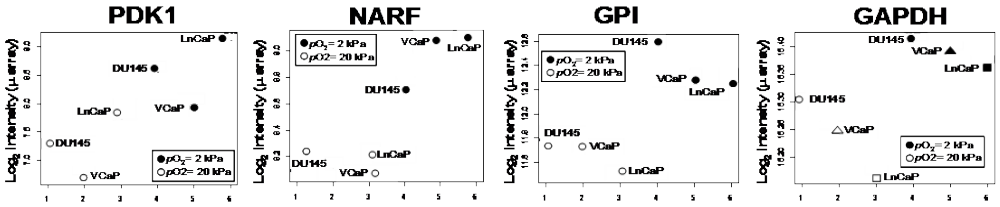
VCaP

Pathway	p-value	# molecules
Organismal Injury and Abnormalities	$7.42x10^{-5}$ – $3.23x10^{-2}$	28
Cancer	$1.13x10^{-4}$ – $3.20x10^{-2}$	184
Hematological Disease	$3.50x10^{-4}$ – $2.93x10^{-2}$	62
Genetic Disorder	$4.42x10^{-4}$ – $3.06x10^{-2}$	346
Respiratory Disease	$4.42x10^{-4}$ – $2.53x10^{-2}$	71

In collaboration with Drs. George Vasmatzis and Farhad Kosari (Mayo Clinic Cancer Center) we compared the analyzed transcriptomes with those of CaP resected tissues previously used to identify prognostic biomarkers (Kosari, Munz et al. 2008). Samples of fresh frozen tissue [(CaP, n=32), benign prostatic tissue adjacent to CaP (BPC, n=40), and benign prostate tissue from CaP–free men (BP, n=28]; and epithelial cells collected from an independent patient set by laser-capture microdissection (LCM) [CaP, n=68;

BPC, n=31; BP, n=11; BPH, n=5]. We compared the data to those from CaP cells cultured at pO_2=20 kPa or pO_2=2 kPa. Notably, hypoxia increased transcript levels for pyruvate dehydrogenase kinase isozyme 1, nuclear prelamin A recognition factor, glucose phosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase in all three cell lines (p<0.05) to levels comparable to those found in primary bulk tissue and LCM isolated cells (p<0.005) **(Figure 2)**.

Figure 2. Different gene expression by CaP cells in hypoxia and normoxia



Expression in LCM and bulk tissue:

symbol	p-Bulk	p-LCM
PDK1	0	0
NARF	0	0.001
GPI	0.4903	0
GAPDH	0.0045	0.004

Transcription profiles revealed different gene expression in cells grown in hypoxia relative to those in normoxia. Interestingly, transcripts for pyruvate dehydrogenase kinase isozyme 1 (PDK1), nuclear prelamin A recognition factor (NARF), glucose phosphate isomerase (GPI), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were overexpressed in hypoxia in all three cell lines (p<0.05), in bulk tissue and LCM isolated cells (p<0.005).

This finding suggests that gene expression in hypoxically cultured cells is more akin to that in tumor cells *in situ* than are cells grown normoxically. Our results challenge the long standing idea suggesting that tumors develop independence of oxygen ["the Warburg effect"; (Stubbs and Griffiths 2010)] that led to the assumption of tumor insensitivity to oxygen. Also our findings add to recent data suggesting that in CaP, tumor–associated hypoxia associates to malignant progression, metastasis, resistance to

Table 2. Hypoxia-associated genes significantly overexpressed in CaP bulk tissue and samples isolated by Laser-capture microdissection

		Bulk tissue		LCM	
Symbol	Name	Ca/N ratio	p value	Ca/N ratio	p value
ACACA	acetyl-Coenzyme A carboxylase alpha	1.5	0	1.2	0.002123
CDCA3	Cell division cycle-associated protein 3	1.7	0	1.5	0
CEP55	centrosomal protein 55kDa	1.8	0	1.4	0
CCNB1	cyclin B1	1.8	0	1.3	0
CKS2	Cyclin-dependent kinases regulatory subunit 2	1.1	0.000043	1.2	0.000001
DLG7	discs, large (Drosophila) homolog-associated protein 5	1.8	0	1.5	0
SLC7A1	High affinity cationic amino acid transporter 1	1.6	0	1.2	0.000023
HMMR	Hyaluronan-mediated motility receptor	2.1	0	1.7	0
HIG2	hypoxia-inducible protein 2	1.5	0	1.2	0.000001
LOX	lysyl oxidase	1.1	0.000044	1.5	0
MMP10	matrix metalloproteinase 10	1.2	0.021521	1.4	0.000066
MCOLN2	mucolipin 2 (cation channel protein)	1.1	0.000332	1.4	0
NLN	neurolysin (metallopeptidase M3 family)	1.2	0	1.2	0.000012
PDLIM5	PDZ and LIM domain 5 (Scaffold protein)	1.1	0	1.2	0.000121
PSD3	pleckstrin and Sec7 domain containing 3	1.1	0.000001	1.2	0
C20orf74	Ral GTPase-activating protein subunit alpha-2	1.3	0	1.2	0.000092
FAM80A	ribosomal modification protein rimK-like family member A	1.7	0	1.2	0.000013
SDK1	sidekick homolog 1, cell adhesion molecule	1.5	0	1.5	0
STC2	Stanniocalcin-2 (secreted)	1.2	0.009053	1.3	0.000059
SOX4	Transcription factor SOX-4	1.2	0	1.2	0.000001
TMEM200A	transmembrane protein 200A	1.3	0	1.3	0.000886
TFF3	trefoil factor 3 (intestinal, stable secretory protein)	1.2	0.001889	1.2	0.001276
UBE2C	Ubiquitin-conjugating enzyme E2 C	1.4	0	1.2	0
UBE2E3	Ubiquitin-conjugating enzyme E2 E3	1.8	0	1.3	0.000079

Abbreviations: Ca, prostate cancer; N, Normal

therapy, and poor clinical outcome (Movsas, Chapman et al. 2000; Vaupel, Kelleher et al. 2001; Chan and Giaccia 2007; Stewart, Ross et al. 2010).

By complementary data mining (Gust, Hofer et al. 2009) we identified 88 known HIF-1 targets and 500 hypoxia associated target genes (Benita, Kikuchi et al. 2009); 23 genes of the conserved core hypoxia signature (Lendahl, Lee et al. 2009); twelve HIF-1 targets tested in CaP (Stewart, Gray et al. 2008); and 708 genes in Ingenuity hypoxia signaling pathway (Ingenuity®Systems). This approach identified 24 hypoxia-associated genes significantly overexpressed in CaP ($p \leq 0.02$), both in bulk tissue and LCM cells **(Table 2)**.

Figure 3 . Three hypoxia-controlled genes are associated with Gleason score and disease prognosis

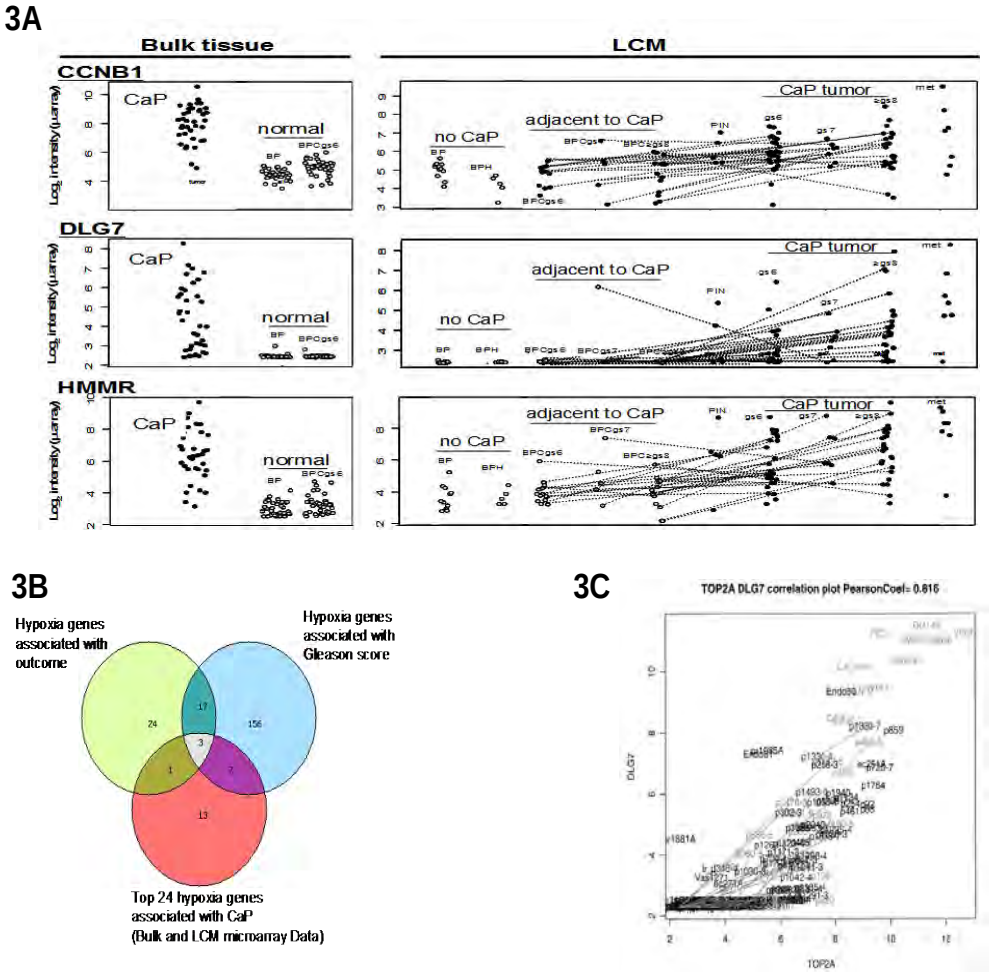


Figure 3A. Abbreviations: BP: benign prostate tissue from men who were free of CaP, BPC: benign prostate tissue in prostates that contained cancer, BPH: Benign prostatic hyperplasia, CaP: prostate cancer, CCNB1: Cyclin B1, DLG7: Disc large (Drosophila) homolog-associated protein 5, HMMR: hyaluronan-mediated motility receptor, LCM: laser-capture microdissection, met: metastasis, PIN: prostatic intraepithelial neoplasia. Figure 3B: Of the 24 hypoxia-controlled genes significantly overexpressed in CaP tissue, three of them: cyclin B1 [CCNB1]; disc large (drosophila) homolog-associated protein 5 [DLG7], and hyaluronan-mediated motility receptor [HMMR] were associated with Gleason score and with disease prognosis. Figure 3C: correlation between the RNA levels of DLG7 and TOP2A in CaP patients (Pearson Coefficient = 0.816)

Among hypoxia-associated genes, the disc large (Drosophila) homolog-associated protein 5 [DLG7], cyclin B1 [CCNB1], and hyaluronan-mediated motility receptor [HMMR] **(Figure 3A)** were associated with Gleason score and disease prognosis **(Figure 3B)**. Since the products of CCNB1 (Gomez, de Las Pozas et al. 2007) and HMMR (Gust, Hofer et al. 2009) genes have been recently identified as molecular markers of CaP progression, our results suggest the potential utility of hypoxia–associated genes as a criterion to identify CaP biomarkers with prognostic value. In additional studies we found a high correlation between DLG7 and DNA topoisomerase 2α (TOP2A) transcript levels (Pearson Coefficient=0.816) **(Figure 3C**). As TOP2A is the strongest predictor of outcome for high-risk CaP (Cheville, Karnes et al. 2008), a predictive value of DLG7 for outcome in men at high-risk CaP can be anticipated.

Recognizing the potential of *in vitro* culture for studies of hypoxia–modulated malignant and survival properties of CaP cells (see above and (Ghafar, Anastasiadis et al. 2003)) we analyzed the transcript levels of DLG7, CCNB1 and HMMR genes in CaP cells grown in normoxia and hypoxia. Hypoxic cells expressed 30 to 60 percent more CCNB1 and DLG7 transcripts. DLG7 is a cell-cycle-regulated (Tsou, Yang et al. 2003), microtubule-associated protein and a Ran GTPase effector involved in mitotic kinetochore fiber stability (Koffa, Casanova et al. 2006). Biological function of DLG7 is compatible with its role in cancer; however, pertinent information on its role in CaP is limited (Tsou, Yang et al. 2003). In one study, DLG7 was detected in nearly 90 percent of transitional cell carcinoma (TCC) of the bladder, but not in benign urological diseases; a higher level of DLG7 was found in recurrent TCC (Chiu, Huang et al. 2002). Overall, our results indicate that the DLG7 expression is higher in CaP and hypoxic CaP cells and correlated with disease outcome. These studies show the feasibility of identifying biomarkers linking CaP hypoxia and prognosis and establishing the contribution of hypoxia-associated genes to CaP progression. Identification of hypoxia-related biomarkers might help identify the patients who could benefit from hypoxia-modulating therapies (Stewart, Ross et al. 2010). These findings constituted the preliminary data for the application entitled "Hypoxia-regulated DLG7 in CaP carcinogenesis and prognosis". This proposal was recently awarded a Treatment Sciences Creativity Award from the Prostate Cancer Foundation and is aimed at validating the role of DLG7 role in tumor progression.

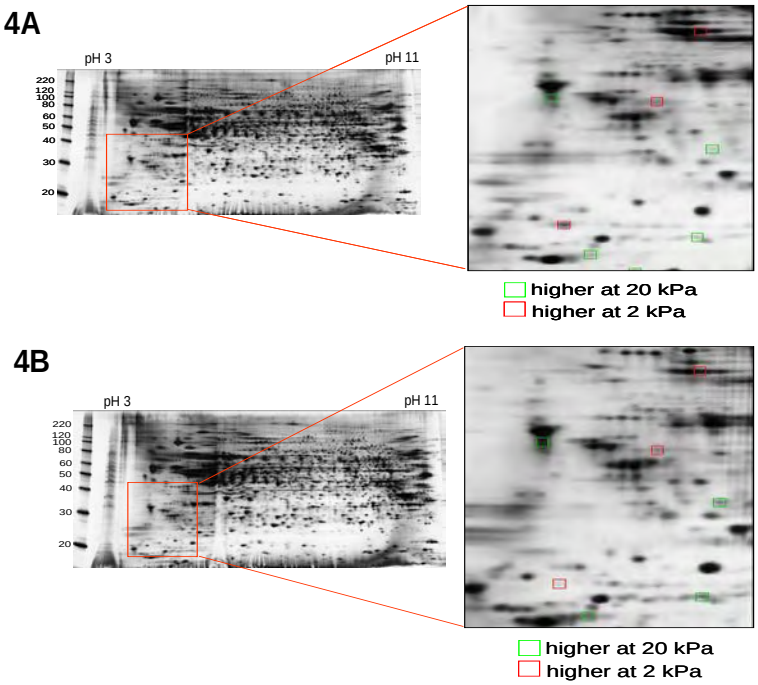
Task 1c. 2-D gel analysis, in gel enzyme digestion and mass spectrometry.

In our preliminary experiments, we observed hypoxia-associated increase in VEGF production in CaP cells and ovarian cancer cells. In addition, we observed differences in spots in CaP cell propagated at pO_2= 2 kPa relative to 20 kPa, as detected by 2-D electrophoresis. This suggests that hypoxia affects protein expression relative to normoxia. To date, identification of specific candidate genes with pO_2-dependent expression in CaP cells in the context of their antigenic relevance has not yet been established.

To characterize the effects of hypoxia on the proteome of CaP cells further, LnCaP and VCaP cells were cultured at pO_2=2 kPa or 20 kPa. Following four or seven days of incubation for LnCaP cells or seven of eleven days for VCaP cells, their lysates were loaded onto nonlinear pH 3−10 strips and subjected to isoelectrofocusing according to manufacturer's instructions (Bio-Rad, Hercules, CA) and published protocols (Desmetz, Bibeau et al. 2008; Sardana, Jung et al. 2008). We ran multiple strips in the first dimension and simultaneous second dimension assuring the highest possible reproducibility. Gels were silver stained, scanned and analyzed by PDQUEST software

(Bio-Rad Laboratories). The proteome revealed multiple spots that differed in intensity and/or position between VCaP cells grown at pO_2=2 kPa and at 20 kPa **(Figure 4)**. Surprisingly, the difference in pO_2 affected the proteome mostly quantitatively (*i.e.*, by change in spot intensity). Using a threshold of fivefold change we found that in VCaP cells cultured for seven days, levels of only 13 proteins decreased and of 4 proteins increased during hypoxia. The results were similar for all cells and culture duration (data not shown). Our results are in line with the reports showing that hypoxia affects expression only of a small fraction of total cellular protein and that the content of total protein is not altered significantly (Koumenis, Naczki et al. 2002; Chen, Shi et al. 2004). In addition, our findings rule out translational modification as an important response to hypoxia in cancer cells.

Figure 4. Oxygen pressure affects VCaP cell proteome



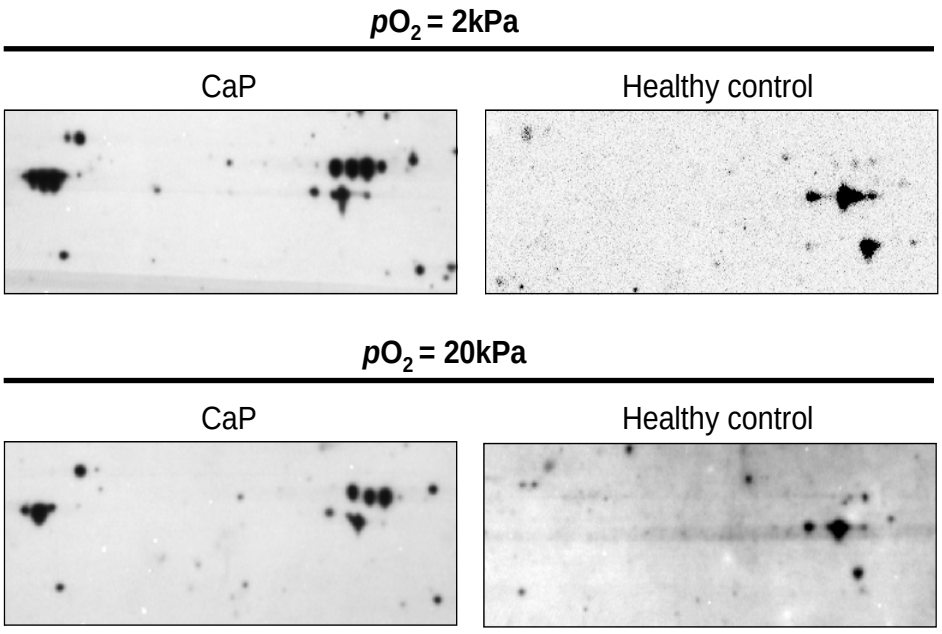
2D SDS electropherograms of lysed VCaP cells cultured at pO_2=2 kPa (Figure 4A) or pO_2=20 kPa (figure 4B) for 7 days were prepared. First dimension: Thirty μg protein were loaded on pH 3-10 NL IPG strips for isoelectric focusing. Second dimension: 10.5-14 % SDS-PAGE gel. Similar results were observed in LnCaP cells (data not shown).

Task 1d. Association between gene-specific changes in mRNA and hypoxic proteome.
In Tasks 1a and 1b we established that hypoxia affects expression of particular genes in CaP and that the effects on proteome are mostly qualitative. However, establishing an association between changes in transcriptome and proteome demands additional effort. Along with similar studies (Koumenis, Naczki et al. 2002; Chen, Shi et al. 2004; Koritzinsky, Seigneuric et al. 2005), we set to identify the most affected protein spots on 2D-gels and find how they compare with the most affected gene transcripts. We sequenced spots from the 2D gels in Task 1e; the spots were selected as potential tumor–associated antigens (TAAs). In a preliminary analysis of six transcripts and proteins, we found no correlation between changes in levels of transcripts and protein (data not shown). The data suggest the possibility that change in protein levels was not

transcription dependent, in line with the findings suggesting that the changes affecting the proteome during hypoxia may be governed by posttranscriptional mechanisms rather than by changes in transcription or translation (Koumenis, Naczki et al. 2002; Chen, Shi et al. 2004; Koritzinsky, Seigneuric et al. 2005).

Task 1e. Identification of CaP antigens by 2D–Western blots.
The finding that patients harbor autoantibodies against tumor antigens has been used as to identify new autoantibody–binding peptides derived from CaP and other tumors (Wang, Yu et al. 2005; Le naour 2006). First we studied the reactivity of autoantibodies in CaP patient plasma. Following published protocols (Desmetz, Bibeau et al. 2008), we prepared total cell lysates of VCaP and LnCaP cells cultured at pO_2=2 kPa or 20 kPa, resolved them by 2D electrophoresis, transferred onto nitrocellulose membranes, and incubated with pooled plasma (1:300) from 25 patients and 25 controls. Plasma of 17 patients with autoimmune diseases (rheumatoid arthritis), 10 with colorectal cancer and 10 with lung cancer was used to validate specificity of potential candidates. Following incubation with goat anti-human Ig–HRP conjugate (1:3000), bound antibodies were detected by chemiluminescence, followed by detection in autoradiographic films. Sera from patients with CaP reacted with numerous spots, some of which were observed in the control groups and thus considered nonspecific **(Figure 5)**.

Figure 5 **. Spontaneous autoantibodies in plasma from newly diagnosed CaP patients against VCaP cells**

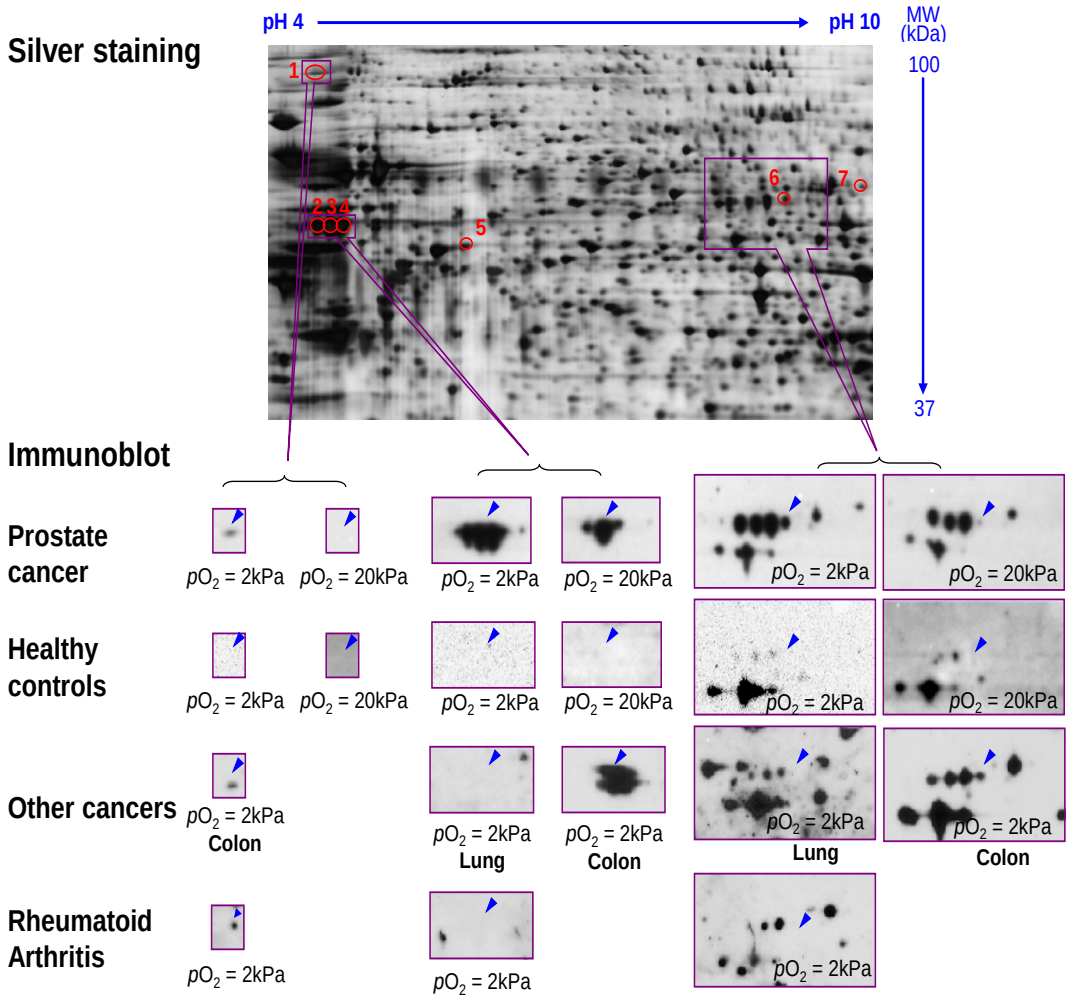


Thirty μg protein were loaded on pH 3-10 NL IPG strips for isoelectric focusing (pH range 4–10 is shown). Second dimension: 10.5-14 % SDS-PAGE gel, transferred to nitrocellulose membranes, incubated with pooled plasma (1:300) from newly diagnosed CaP patients (n=5; total of 4 pools) or age matched non-cancerous controls (n=5; total of 4 pools). Following incubation with chicken anti-human IgG-HRP, spots were identified by chemiluminescence.

Sera from CaP patients specifically bound to seven spots **(Figure 6)**; four were hypoxia-specific. All selected spots were excised from the gel, trypsin-digested, and analyzed by

MALDI-TOF mass spectrometry. We identified them as heat shock 70 kDa protein 4; 60 kDa heat shock protein; protein disulfide isomerase A3; heterogeneous nuclear ribonucleoprotein L; U1 small nuclear ribonucleoprotein 70kDa and leucine-rich repeat-containing protein 47. With the exception of the latter molecule, identified proteins have been identified or validated as TAAs before [see references in **(Table 3**)]. However, to the best of our knowledge none of the proteins has been validated as a TAA in CaP.

Figure 6. Identification of hypoxia-specific protein spots from CaP patients



VCaP cells were cultured at pO_2=2 kPa or pO_2=20 kPa for 7 days and cell lysates were prepared. Thirty μg protein were loaded on pH 3-10 NL IPG strips for isoelectric focusing. Second dimension: 10.5-14 % SDS-PAGE gel. One set of gels was silver stained and other set was transferred to nitrocellulose membranes, incubated with pooled plasma (1:300) from newly diagnosed CaP patients (n=5; total of 4 pools), age matched non-cancerous controls (n=5; total of 4 pools), patients with other cancers [colon (n=10); lung (n=10)] and autoimmune disease [rheumatoid arthritis (n=20)]. Following incubation with chicken anti-human IgG-HRP, spots were identified by chemiluminescence. The arrowheads indicates protein spots of interest.

Table 3. Potential TAAs identified in VCaP cells

Spot number	Protein name	Hypoxia specific	Accession number	MW (kDa)	Peptides matched	TAA (Refs)
1	Heat shock 70 kDa protein 4	yes	P34932	94.3	28-39	Esophageal (Zhang, Wang et al. 2011), hepatocellular carcinoma (Takashima, Kuramitsu et al. 2006; Looi, Nakayasu et al. 2008)
2	60 kDa heat shock protein	no	P10809	61.3	43-63	Breast (Desmetz, Bibeau et al. 2008), hepatocellular carcinoma (Looi, Nakayasu et al. 2008), colorectal (He, Wu et al. 2007), oral (Castelli, Cianfriglia et al. 2001), gastric lymphoma (Takenaka, Yokota et al. 2004)
3	60 kDa heat shock protein	no	P10809	61.3	59-80	
4	60 kDa heat shock protein	no	P10809	61.3	50-105	
5	Protein disulfide isomerase A3	yes	P30101	56.8	16-26	Breast (Desmetz, Bibeau et al. 2008; Hamrita, Chahed et al. 2008), hepatocellular carcinoma (Looi, Nakayasu et al. 2008)
6	Heterogeneous nuclear ribonucleoprotein L	yes	P14866	64.1	18-52	Acute leukemia (Cui, Li et al. 2005), healthy (Li, Zhao et al. 2006)
6	U1 small nuclear ribonucleoprotein 70kDa	yes	P08621	51.4	11-52	Lymphoma (Cha, Kwak et al. 2006)
7	Leucine-rich repeat-containing protein 47	yes	Q8N1G4	63.5	16-16	

Interestingly, the sequence of spots 2, 3 and 4 in Figure 6 corresponded to the 60 kDa heat shock protein, identified by us as a hypoxia–insensitive TAA. As there are three Hsp60 isoforms (Raulston, Paul et al. 1998), it is likely that we identified the three isoforms as potential TAAs. Additional research will clarify the relevance of Hsp60 isoforms as relevant TAAs in CaP. Lysates from LnCaP cells grown at pO_2=2 kPa interacted with specific antibodies in plasma from CaP patients (data not shown); however, overall reactivity was lower than in VCaP cells. Interestingly, a series of spots consistent with spots 2-4 in VCaP cells was also recognized by plasma from patients blotted against lysates from LnCaP cells. It is evident from those experiments that hypoxic LnCaP cells exhibit similar reactivity to hypoxic VCaP cells. We are currently sequencing identified proteins from LnCaP cells with the expectation that they will validate the spots in VCaP cells and identify additional novel hypoxia-sensitive TAAs.

Among the most conspicuous spots recognized by plasma from CaP patients in VCaP lysates was spot 6; this hypoxia-sensitive spot was strongly reactive with the pooled sera of CaP patients **(Figure 6)**. This spot contains the U1 small nuclear ribonucleoprotein 70kDa and the heterogeneous nuclear ribonucleoprotein L. Both these proteins have been identified as TAAs (Cui, Li et al. 2005; Cha, Kwak et al. 2006; Li, Zhao et al. 2006); their validation in CaP is our next immediate priority. For validation we will narrow the pH range of isoelectric focusing from 7 to 10 and use specific antibodies to confirm the identity of the molecules. Protein spot 6 exhibited little or no reactivity with sera of healthy controls, lung cancer or rheumatoid arthritis, but we detected some reactivity (in a hypoxia-dependent manner) with plasma from colorectal cancer (CRC) patients. The

relevance of hypoxia has been recognized in CRC (Waldner and Neurath 2010) and numerous CRC-associated-TAAs have been identified (Reuschenbach, von Knebel Doeberitz et al. 2009); however, establishing the relevance of hypoxia-sensitive U1 small nuclear ribonucleoprotein 70kDa and heterogeneous nuclear ribonucleoprotein L is a new and interesting aspect in CRC as it could expand the use of hypoxia to the identification of TAAs in other tumors.

Summary of key research accomplishments related to Task 1 (as they relate to the proposed sub Tasks):

Task 1a. To propagate LnCaP and VCaP cells under pO_2–controlled conditions:

- Hypoxic LnCaP and VCaP cells proliferate more effectively than at standard cell culture conditions.
- Hypoxic cells secrete more VEGF.

Task 1b. cDNA gene microarrays and data analysis:

- Hypoxia induced overexpression of molecules involved in intracellular signaling networks in cancer and in urologic diseases in comparison to normoxic cells.
- Hypoxia increased transcript levels for some genes in cell lines to levels comparable to those in CaP tissue.
- Hypoxia-associated the disc large (Drosophila) homolog-associated protein 5 [DLG7], cyclin B1 [CCNB1], and hyaluronan-mediated motility receptor [HMMR] genes were significantly overexpressed in CaP and were associated with Gleason score and disease prognosis.
- Hypoxic cells expressed 30 to 60 percent more CCNB1 and DLG7 transcripts.

Task 1c. 2-D gel analysis, in gel enzyme digestion and mass spectrometry:

- The change in pO_2, affected the proteome mostly quantitatively (*i.e.*, by change in spot intensity).

Task 1d. Association between gene-specific changes in mRNA and hypoxic proteome:

- There was no correlation between changes in protein levels and mRNA induction among a group of select genes tested.

Task 1e. Identification of CaP antigens by 2D–Western blots

- Protein lysates from cells exposed to hypoxia revealed novel potential tumor associated antigens (TAAs) (currently under validation) in sera from CaP patients (heat shock 70 kDa protein 4; protein disulfide isomerase A3; heterogeneous nuclear ribonucleoprotein L; U1 small nuclear ribonucleoprotein 70kDa and leucine-rich repeat-containing protein 47).

Task 2. Validate differentially expressed molecules in CaP in association with tissue hypoxia

Approach: The presence of a hypoxic cancer microenvironment correlates with increased tumor invasiveness, metastases, resistance to radio- and chemotherapy, and poor clinical outcome (Vaupel, Kelleher et al. 2001; Overgaard 2007). It is well established that CaP

cells are found under hypoxic conditions *in vivo* (Movsas, Chapman et al. 2001) and that numerous proteins are modified in their expression by hypoxia (Koritzinsky, Seigneuric et al. 2005). Although many endogenous markers have been associated with the hypoxia response in cancer they are not all unregulated in primary CaP tissue (Stewart, Gray et al. 2008). This may be because the evaluation of potential markers has not been made taking into consideration the hypoxic environment in first place. Task 2 is aimed at assessing the expression of select candidate genes identified in Task 1 in CaP tissue. Real time PCR and RNA *in situ* hybridization added to immunodetection will allow detection of specific candidate genes in CaP tissue.

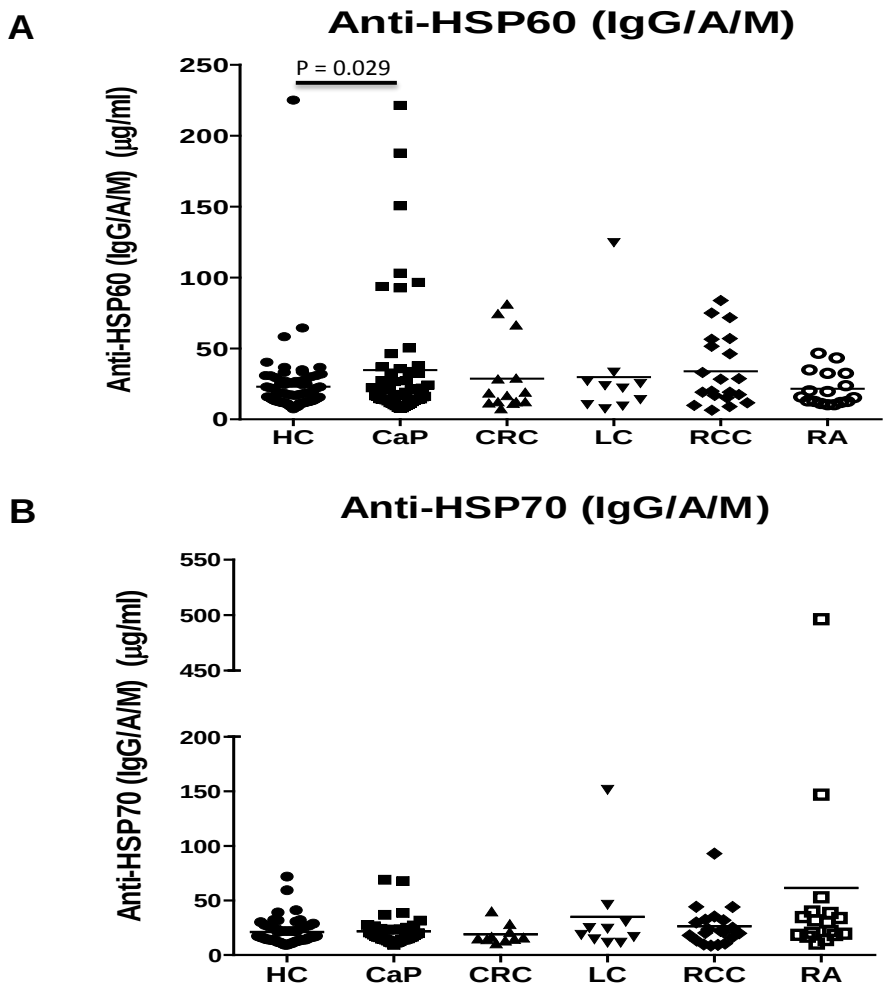
The proposed sub Tasks are:
2a. RNA extraction and real time quantitative PCR in CaP tissue (months 13-16)
2b. mRNA *in situ* hybridization (months 16-22)
2c. Immunohistochemistry Staining in CaP tissue (months 16-24)

Note: Because of relocation to the University of Mississippi Medical Center (UMMC) in September 2011, this task was re-purposed. I requested a no-cost extension to the PCRP-CDMRP. Approval was received on May 2012, extending the period of performance from: 15 May 2010 - 14 June 2012 (research ends 14 May 2012) to 15 May 2010 - 14 June 2013 (research ends 14 May 2013). Based on resources availability, we decided to focus on the validation of autoantibodies selected in Aim 1 in patient plasma and correlation with tumor level expression for those candidates.

Frequency of Autoantibodies in the plasma of CaP patients: We performed ELISA tests to determine the frequency of autoantibodies for two of the three selected potential TAAs identified by 2D–Western blots: HSP60 and HSP70. The sera of CaP patients (n=54), healthy controls [males (n=76); females (n=6)], colorectal cancer (n=14), renal cell carcinoma (n=20), lung cancer (n=10), and rheumatoid arthritis (n=17). The frequencies of IgG/A/M for HSP60 were quantified using commercial kits [Anti-HSP60 IgG/A/M (cat# ADI-EKS-650) and Anti-HSP70 IgG/A/M cat# ADI-EKS-750)] from Enzo Life Sciences (Plymouth Meeting, PA). This assay allows for reproducible, accurate, and precise determination of IgG, IgA and IgM antibodies (total) to the analyte of interest in plasma. Recombinant human protein (e.g. HSP60 or HSP70) is bound to the wells of the plate to bind anti-human antibodies for the analyte of interest present in plasma. The captured anti-human antibodies are detected with a HRP conjugated goat polyclonal antibody specific for human IgG, IgA, and IgM molecules. The results are expressed in proportion to the amount of captured anti-human antibodies. The frequency of HSP60 autoantibodies **(Figure 7A)** were 13.0% (7/54) in CaP (average ± SD = 34.67 ± 43.94 µg/ml), compared to1.3% (1/76) in healthy controls (average ± SD = 22.96 ± 25.77 µg/ml); 7.14% (1/14) in colorectal cancer (average ± SD = 28.66 ± 25.52 µg/ml); 10.0% (1/10) in lung cancer (average ± SD = 29.72 ± 34.42 µg/ml); 5.0% (1/20) in renal cell carcinoma (average ± SD = 33.82 ± 24.20 µg/ml); and 0% (0/17) in rheumatoid arthritis (average ± SD = 21.52 ± 11.93 µg/ml). These data suggest that HSP60 autoantibodies may be elevated in the CaP patient plasma
Next, the frequency of HSP70 autoantibodies (identified as a hypoxia-specific TAA) was analyzed. TAAs frequency for HSP70 **(Figure 7B)** were 5.3% (2/38) in CaP (average ± SD = 21.81 ± 13.06 µg/ml), compared to 3.7% (2/54) in healthy controls (average ± SD = 21.10 ± 11.80 µg/ml); 7.14% (0/10) in colorectal cancer (average ± SD = 19.01 ± 8.81

µg/ml); 20.0% (2/10) in lung cancer (average ± SD = 34.93 ± 42.21 µg/ml); 5.0% (1/20) in renal cell carcinoma (average ± SD = 26.40 ± 18.97 µg/ml); and 17.65% (3/17) in rheumatoid arthritis (average ± SD = 61.49 ± 116.3 µg/ml). TAAs for HSP70 were not elevated in CaP patients relative to healthy controls; and even more, higher autoantibodies frequencies were found in other tumors and in rheumatoid arthritis (in agreement with data from 2D-Westerm blot), a non-cancer-related inflammatory disease. This result contrasts with previous findings suggesting HSP70 as a potential TAA in esophageal (Zhang, Wang et al. 2011), and hepatocellular carcinoma (Takashima, Kuramitsu et al. 2006; Looi, Nakayasu et al. 2008)

Figure 7. Autoantibodies in the Plasma of CaP Patients

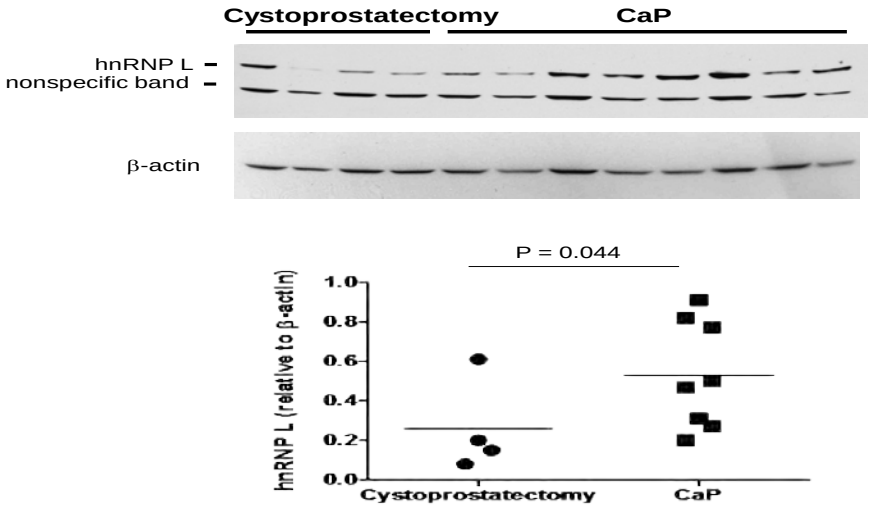


Autoantibodies to HSP60 **(Figure 7A)** and HSP70 **(Figure 7B)** were quantitated in the plasma of prostate cancer (CaP; n = 54); healthy controls (HC; n = 76); colorectal cancer (CRC; n = 14); lung cancer (LC; n = 10); renal cell carcinoma (RCC; n = 20); and rheumatoid arthritis (RA; n = 17). Plasma samples were diluted to 1/1000. The cutoff of reactivity was defined as the mean of sample plus 2 folds of standard deviation from normal plasma. Comparisons were done using t- test, one-tail. The level of significance was set at $P < 0.05$.

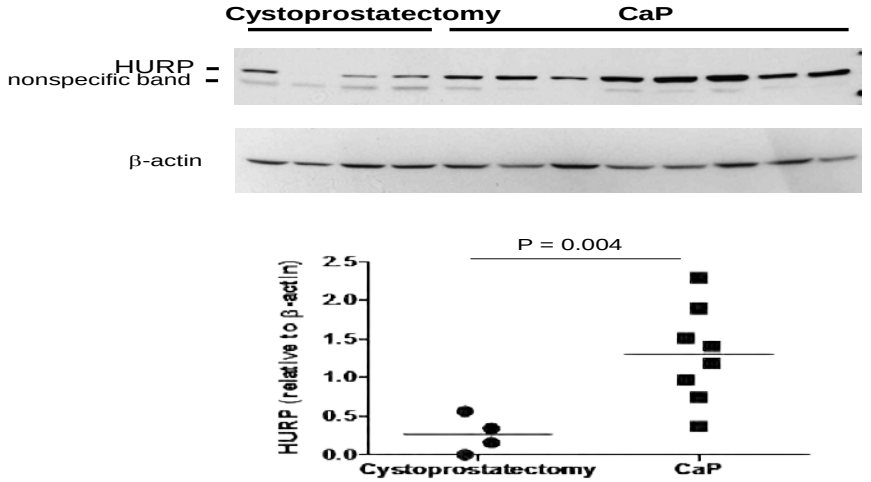
Overall, this data confirms HSP60 as a potential TAA in CaP. The results are in agreement with reported findings in other tumors (Castelli, Cianfriglia et al. 2001; Takenaka, Yokota et al. 2004; He, Wu et al. 2007; Desmetz, Bibeau et al. 2008; Looi, Nakayasu et al. 2008) demonstrating that HSP60 can elicit a humoral response. Our data represents the first one to suggest a specific humoral response against HSP60 in CaP. Further data evaluation in an independent validation group is needed to evaluate the

Figure 8. Protein levels in CaP tumor lysates

A hnRNP L



B HURP



Protein lysates were prepared from prostate frozen tissue obtained from CaP patients (n=8) and cystoprostatectomy patients (n=4) used as controls. Thirty μg protein were resolved in a 10.5-14 % SDS-PAGE gel; and transferred to a nitrocellulose membrane, blotted with anti –hnRNP L (1/5000) **(Figure 8A)**, –HURP (1/100) **(Figure 8B)** and –beta (β) actin (1/5000) antibodies. Following incubation with the respective IgG-HRP, bands were identified by chemiluminescence. The relative intensities of the bands were quantified using the Image J Software, and all the values were normalized to the intensities of the respective β-Actin signal. Results are expressed as the normalized optical density (OD) for individual patients. The mean OD for each experimental group is also shown. Comparisons were done the using the Mann–Whitney U test. The level of significance was set at $P < 0.05$.

performance and diagnostic value of HSP60 autoantibodies. For this purpose we are currently collaborating with Faculty at the Department of Urology and Pathology at UMMC. Blood and plasma samples are being collected from CaP patients recruited from Urology Clinics at the UMMC. An aliquot of plasma is secured for TAAs studies. Currently, we are in the process of acquiring the reagents for detecting antibodies to hnRNP L; its validation is critical in order to test the value of this, hypoxia-specific, as a potential TAA.

To examine the possibility that the reactivity of CaP sera is due to an elevated expression of selected TAAs, we prepared protein lysates from tumors obtained from eight CaP patients. Protein lysates from four control cystoprostatectomy patients were used as controls. Separated proteins were transferred onto a nitrocellulose membrane and blotted against an anti-human hnRNP L monoclonal antibody (Abcam, cat# ab6106). The results were normalized to beta (β)-actin (Novus, cat# NB600-501) and the ratio between hnRNP L and beta-actin was calculated to normalize the data. Normalized ratios were compared between controls and CaP tumors. The level of hnRNP L protein was significantly higher ($p < 0.05$); with a 2.04 fold increase compared to control prostate tissue **(Figure 8A)**. These data suggest that protein expression for at least one of the selected potential TAAs under current validation may be elevated in CaP. These results warrant the study of gene expression in conjunction with a histopathology evaluation of CaP tissue. As part of the Prostate Cancer Foundation (PCF) awarded project "Hypoxia-regulated expression of DLG7 gene in prostate cancer prognosis and progression", assessment the relationship of DLG7 expression and cancer-specific outcomes is a most relevant Task. We applied a similar strategy to explore the expression of the DLG7 gene product, hepatoma up-regulated protein (HURP) in CaP tissue with high RNA expression for DLG7, identified by cDNA microarrays. The level of HURP protein (detected by anti-HURP antibodies, cat# ab79870) was significantly higher ($p < 0.005$); with a 4.89 fold increase compared to control prostate tissue **(Figure 8B)**; therefore, suggesting that gene and protein expression can be associated in our experimental groups.

Analysis of the association of transcript and protein levels with clinical parameters will follow. In November 2011 I was offered a Research Collaborator position at the Department of Hematology, Mayo Clinic, Rochester, MN. The appointment allows ongoing funded research projects between my lab and Mayo Clinic researchers to continue, and to develop and maintain future investigative efforts. In relation to this project, validation of candidate TAAs in plasma and CaP tissue from patients belonging to the Mayo's prostate SPORE is in place. For this last purpose there is a currently active IRB protocol (Mayo IRB#10-000306). Processing and transferring of frozen tissue for RNA analysis; and paraffin embedded tissue sections for immunostaining will be performed by Drs. George Vasmatzis, John Cheville, and Farhad Kosari at Mayo's Center for individualized Medicine.

Key research accomplishments for Aim2

- Three potential TAAs; two hypoxia-specific (HSP70 and hnRNP L) and one hypoxia-nonspecific (HSP60) were selected for validation.
- The frequency of HSP60 autoantibodies was elevated in the CaP patient plasma.
- The frequency of HSP70 autoantibodies was not elevated in CaP patients relative to healthy controls; even more, higher autoantibodies frequencies were found in other tumors and in rheumatoid arthritis.

- Protein expression for hnRNP L (detected by Western Blot technique) was elevated in CaP tissue. Similarly, protein expression for HURP, another hypoxia-specific protein, was elevated in CaP tissue.

Key research accomplishments for Aim1 and Aim2:

- Hypoxic LnCaP and VCaP cells proliferate more effectively than at standard cell culture conditions.
- Hypoxic cells secrete more VEGF.
- Hypoxia induced overexpression of molecules involved in intracellular signaling networks in cancer and in urologic diseases in comparison to normoxic cells.
- Hypoxia increased transcript levels for some genes in cell lines to levels comparable to those in CaP tissue.
- Hypoxia-associated the disc large (Drosophila) homolog-associated protein 5 [DLG7], cyclin B1 [CCNB1], and hyaluronan-mediated motility receptor [HMMR] genes were significantly overexpressed in CaP and were associated with Gleason score and disease prognosis.
- Hypoxic cells expressed 30 to 60 percent more CCNB1 and DLG7 transcripts.
- The change in pO_2, affected the proteome mostly quantitatively (*i.e.*, by change in spot intensity).
- There was no correlation between changes in protein levels and mRNA induction among a group of select genes tested.
- Protein lysates from cells exposed to hypoxia revealed novel potential tumor associated antigens (TAAs) (currently under validation) in sera from CaP patients (heat shock 70 kDa protein 4; protein disulfide isomerase A3; heterogeneous nuclear ribonucleoprotein L; U1 small nuclear ribonucleoprotein 70kDa and leucine-rich repeat-containing protein 47).
- Three potential TAAs; two hypoxia-specific (HSP70 and hnRNP L) and one hypoxia-nonspecific (HSP60) were selected for validation.
- The frequency of HSP60 autoantibodies was elevated in the CaP patient plasma.
- The frequency of HSP70 autoantibodies was not elevated in CaP patients relative to healthy controls; even more, higher autoantibodies frequencies were found in other tumors and in rheumatoid arthritis.
- Protein expression for hnRNP L (detected by Western Blot technique) was elevated in CaP tissue. Similarly, protein expression for HURP, another hypoxia-specific protein, was elevated in CaP tissue.

Reportable outcomes

Articles

1. **Gomez, C.R.,** Knutson, G., Bulur, P., Schreiber, C., Vuk-Pavlovic S. Age-dependent response of bone marrow cells to hyperbaric oxygen. BMC Medicine. DOI: 10.1007/s10522-012-9373-8
2. Nomellini, V., Mahbub, S., **Gomez, C.R.,** and Kovacs, E.J. Dysregulation of neutrophil CXCR2 and pulmonary endothelial icam-1 promotes age-related pulmonary inflammation. 2012. Aging Dis. 3:234-47
3.

Abstracts

1. **Gomez, C.R.,** Kosari, F., Schreiber, C.A., Knutson G.J., Vasmatzis, G.,Vuk-Pavlović S. 2010. Hypoxic exposure as a novel strategy to identify tumor-associated antigens in prostate cancer. Tumor Immunology: Basic and Clinical Advances. Miami Beach, FL
2. **Gomez, C.R.,** Knutson G.J., Schreiber, C.A., Kosari, F., Vasmatzis, G.,Vuk-Pavlović S. 2011. Hypoxia affects gene expression and proteome of prostate cancer cells. 102th AACR Annual Meeting. Orlando, FL
3. **Gomez, C.R.,** Kosari, F., Schreiber, C.A., Knutson G.J., Vasmatzis, G., Vuk-Pavlović S. 2011. Hypoxic cell culture for more effective cancer vaccines. International Society for Applied Biological Sciences Conference on Forensic Genetics (ISABS). Bluesun hotel ELAPHUSA, Bol, Island Brač, Croatia
4. **Gomez, C.R.,** Munz, J.M., Kosari, F., Karnes R.J., Cheville, J., Ida, C.M., Sebo, T.J., Nair, A.A., Tang, H., Vasmatzis, G., Vuk-Pavlović S. 2011. Prognostic value of hypoxia-controlled genes in high-risk prostate cancer. Eighteen Annual Prostate Cancer Foundation Scientific Retreat. Incline Village, Lake Tahoe, NV
5. **Gomez, C.R.,** Schreiber, C.A., Knutson G.J., Charlesworth, M.C., Vuk-Pavlović S. 2011. Targeting hypoxia for more effective immunotherapy of prostate cancer. Molecular Targets and Cancer Therapeutics AACR-NCI-EORTC International Conference. San Francisco, CA
6. **Gomez, C.R.,** Kosari, F., Munz, J.M., Schreiber, C., Knutson, G., Charlesworth, C., Karnes, R. J., Cheville, J., Vasmatzis, G., Vuk-Pavlovic, S. 2012. Prognostic value of hypoxia-associated genes in prostate cancer. Advances in Prostate Cancer Research. Orlando, FL
7. **Gomez, C.R.,** Munz, J.M., Kosari, F., Karnes R.J., Cheville, J., Ida, C.M., Sebo, T.J., Vasmatzis, G., Vuk-Pavlović S. 2012. Targeting hypoxia for the identification of novel biomarkers for prostate cancer. 103th AACR Annual Meeting. Chicago, IL
8. Ma T., Elkhattouti A., Kosari F., Karnes R.J., Cheville, J., Vasmatzis, G., Vuk-Pavlović S., **Gomez, C.R.,** 2012. Hypoxia-reactive proteins as potential tumor-associated antigens in prostate cancer. Nineteen Annual Prostate Cancer Foundation Scientific Retreat. Carlsbad, CA
9. Ma T., Elkhattouti A., Kosari F., Karnes R.J., Cheville, J., Vasmatzis, G., Vuk-Pavlović S., **Gomez, C.R.** 2013. Hypoxia as a tool for identifying prostate cancer-associated antigens. Carlsbad, CA. 104th AACR Annual Meeting. Washington, DC
10. Ma T., Elkhattouti A., Vuk-Pavlović S., **Gomez, C.R.** 2013. Identification and validation of hypoxia-specific tumor associated antigens in prostate cancer. 15th International Congress on Immunology. Milan, Italy
11. **Gomez, C.R.,** Kosari F., Vasmatzis, G., Ma T., Vuk-Pavlović, S., Vijayakumar, S. 2013. Oxygen Tension as a Modifier Agent of Prostate Cancer Cells: Potential for the Identification of Novel Tumor Microenvironment-relevant Imaging Probes. The 6th Annual World Molecular Imaging Congress. Savannah, GA
12. Elkhattouti, A., Ma, T., Kent, J.R., Espinoza, I., **Gomez, C.R.** 2013. Role of Hepatoma Up-regulated Protein (HURP) in prostate cancer tumorigenesis. AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics. Boston, MA

Funding Applied

1. Tittle: Hypoxia-regulated DLG7 in prostate cancer carcinogenesis and prognosis (P.I.). Date: Dec 2010. Submitted to: Prostate Cancer Foundation. Treatment Sciences Creativity Awards. Level of Funding: $300,000. Status: Funded. Project

goals: We found the transcripts of the discs large homolog-associated protein 5 (DLG7), a hypoxia-regulated gene, overexpressed in human primary prostate cancer and human prostate cancer cell lines. The overall goal of the proposed studies is to validate the role of DLG7 role in tumor progression. Specific aims: 1) To measure the levels of DLG7 transcripts in resected CaP tissues and study the association with survival. 2) To overexpress DLG7 in prostate cells (normal epithelium and tumorigenic cells) and compare tumorigenesis in the context of hypoxia. Key personnel receiving salary support from this project: 50% effort CR Gomez. 100% effort postdoctoral fellow

2. Tittle: A Method for Prognosis of Prostate Cancer Based on Cellular Markers of Hypoxia (P.I.). Date: Jan 2011. Submitted to: Mayo Clinic Center for Translational Science Activities (CTSA) - Novel Methodology Development Award (NMDA). Level of Funding: $50,000. Status: Not funded.
3. Title: Hypoxia enhances prostate cancer radioresistance by promoting cancer cell stemness (P.I.). Date: March 2012. Funding agency: BD Biosciences Research Grant Program-Stem Cell Grant. Time commitment: 5%. Performance period: 07/01/12 – 07/01/14. Level of funding: $10,000. Status: Not funded. Project goal: test the hypothesis that a low oxygen environment (where radiation is less effective due to the lack of the oxygen effect) increases the fraction of cancer stem cells in the population giving thus rise to a more resistant tumor. Key personnel receiving salary support from this project: 10% effort flow cytometry core personnel.
4. Title: Mississippi Prostate Cancer HBCU Undergraduate Research Training Program. (Program Coordinator and Mentor). Date: June 2012. Funding agency: Collaborative Undergraduate HBCU Student Summer Training Program Award from the Department of Defense; CDMRP, Prostate Cancer Research Program. Performance period: 04/01/13 – 03/30/15. Level of Funding: $185,000. Status: Not Funded. Project goals: Aim 1: To recruit 4 undergraduate trainees per year from Tougaloo College; Aim 2: To develop their skills through a comprehensive training curriculum in CaP research at UMMC-Cancer Institute and; Aim 3: To track and coach trainees on their progress towards becoming biomedical CaP researchers. Key personnel receiving salary support from this project: Srinivasan Vijayakumar, M.D. 0.12 Calendar months (Principal Investigator), Christian R. Gomez, Ph.D. 0.36 Calendar months (Program Coordinator and Mentor).
5. Title: A gene panel predictive of outcome in men at high-risk prostate cancer: Prognostic performance in African Americans. Funding agency: Bayer-Grants4Targets. Time commitment: 5%. Performance period: 06/01/12 – 06/01/14. Level of funding: $150,000. Status: Not funded. Project goal: test the hypothesis that a panel of hypoxia-sensitive genes and TAAs identified in Caucasians has prognostic value in patients from other races with diagnosed CaP and in other cancers. Key personnel receiving salary support from this project: 100% effort postdoctoral fellow.
6. Title: Mississippi Prostate Cancer HBCU Undergraduate Research Training Program. (Program Coordinator and Mentor). Date: August 2013. Funding agency: Collaborative Undergraduate HBCU Student Summer Training Program Award from the Department of Defense; CDMRP, Prostate Cancer Research Program. Performance period: 04/01/14 – 03/30/16. Level of Funding: $185,000. Status: Under review. Project goals: Aim 1: To recruit 4 undergraduate trainees

per year from Tougaloo College and Jackson State University; Aim 2: To develop their skills through a comprehensive training curriculum in CaP research at UMMC-Cancer Institute and; Aim 3: To track and coach trainees on their progress towards becoming biomedical CaP researchers. Key personnel receiving salary support from this project: Kounosuke Watabe 0.30 Calendar months (P.I.), Christian R. Gomez, Ph.D. 0.34 Calendar months (Program Coordinator and Mentor).

7. Title: Role of Hepatoma Up-regulated protein (HURP) in prostate cancer (Mentor). Date: August 2013. Funding agency: Post-Doctoral Fellowship Award from the Department of Defense; CDMRP, Prostate Cancer Research Program. Performance period: 04/01/14 – 03/30/16. Level of Funding: $115,000. Status: Under review. Project goals: Aim 1: To determine the HURP-mediated effects on PCa tumorigenesis. Aim 2: To study if the tumorigenic effects of HURP in PCa are mediated by HURP's stemness-enhancing properties. Key personnel receiving salary support from this project: Abdelouahid El Khattouti, Ph.D. 12 Calendar months (P.I.).

Research opportunities

In November 2011 the PI was offered a Research Collaborator position at the Department of Hematology, Mayo Clinic, Rochester, MN (see appended appointment notification letter). The appointment allows continuing ongoing funded research projects between his lab and Mayo Clinic researchers, and to develop and maintain future investigative efforts. At UMMC, the PI is beginning experiments aimed at validating our findings in the context of the health disparities issue. For this purpose, transfer of materials and data between his lab at UMMC and others throughout Mayo Clinic is critical. This appointment will be beneficial both for the discovery of novel tumor biomarkers and for the development of better therapy for CaP.

In May 2012 the PI was offered an appointment at The School of Graduate Studies in the Health Sciences, UMMC, Jackson, MS (see appended appointment notification letter). This appointment allows me to receive graduate students in my lab and participate in other academic activities associated to the Graduate School.

In December 2012 the PI was offered an Associate Professor Appointment at the Department of Radiation Oncology, UMMC, Jackson, MS (see appended appointment notification letter). The appointment allows developing research on prostate cancer hypoxia-controlled molecules as biomarkers on the context of radioresistance. In one of these studies Dr. Ahmed Ejaeidi M.D., a graduate student in my lab, enrolled in the Ph.D. Program in Pathology, UMMC, is developing a joint project with me and Dr. Srinivasan Vijayakumar M.D., Chair of the Radiation Oncology Department at UMMC. Part of his dissertation project will involve evaluating the expression of stem cancer stem in biopsies from CaP patients who underwent radiation therapy. We will perform a retrospective review of clinical records of CaP patients with localized disease who underwent external beam radiotherapy for primary treatment of localized CaP. The expression of stemness factors and hypoxia controlled genes will be determined by real-time PCR in core biopsies collected previously to treatment. Furthermore, the association between the presence of stem-like cells and tumor volume (assumed as a partial indicator of hypoxia) will be explored. Expression of stemness markers and hypoxia controlled genes is expected elevated in non-responders. Likewise, tumor volume would correlate with the

numbers of stem-like cancer cells and the gene expression of stemness markers and hypoxia-controlled genes.
In June 2013 the PI was offered a Research Faculty appointment at the G.V. (Sonny) Montgomery VA Medical Center, Jackson, MS (see appended appointment notification letter). In collaboration with Dr. Chadwick Huckabay M.D., faculty at the VA, we will determine whether DLG7 presence in Caucasians can be used as a prognostic indicator for outcomes in other populations with CaP. Also, we will verify that there is not a difference between the expression of DLG7 in veterans vs. non-veterans. Access to the VA's archival database, a well-known resource for long term follow-ups, will allow us to develop future projects to study potential biomarkers for CaP, and eventually to test their potential prognostic and theragnostics value.

Employment applied
In September 2011 the PI accepted a tenure–track Associate Professor position at the Department of Pathology, University of Mississippi Cancer Center, Jackson, MS (see appended welcoming letter from Dr. James Keeton, Vice Chancellor and Dean) A seeding/Start-up Grant with an estimated performance period of 5 years (09/26/11 – 09/26/16) was received. Level of funding: $375,000 in direct costs. These funds cover lab equipment, supplies and personnel salaries. Added to external, peer-reviewed funding held by the PI from the DOD (PCRP W81XWH-10-1-0225) and the PCF (2011 Creativity Award) make a total close to $1.000.000 of combined independent research funding. The general research interests focus of the lab is on modulating immunity to offset the effects of disease and aging. The overall goal is to develop basic research with immediate translational potential and technological applicability in cellular immunotherapy of cancer, immune reconstitution, and regenerative medicine. Ongoing efforts are to develop more effective cancer immunotherapy, particularly immunotherapy of CaP. For more information please visit the following web site: http://cancerinstitute.umc.edu/profiles/gomez_christian.html

Conclusion
We are studying the ways to optimize the effectiveness of therapeutic whole–cell CaP vaccines by tumor–associated hypoxia as a relevant determinant of tumor antigenicity. Our results show that in cultured human CaP cells hypoxia modifies expression of genes associated with cancer and urologic disease to levels comparable to those in resected human CaP tissue. These results suggest that gene expression in hypoxically cultured cells is more akin to that in tumor cells *in situ* than are cells grown normoxically. We studied the transcriptome in human primary CaP tissue. Transcripts of hypoxia–associated genes DLG7, CCNB1 and HMMR were overexpressed and associated with Gleason score and with disease prognosis; this suggests their potential as CaP biomarkers with prognostic value. 2D-gel electrophoresis experiments confirmed previous findings indicating that hypoxia affects the proteome mostly quantitatively (*i.e.*, by change in spot intensity). Nonetheless, protein lysates from CaP cells exposed to hypoxia revealed novel potential TAAs thus suggesting the relevance of antigenic landscape of hypoxic proteome. The value of selected candidates (i.e. HSP60 and hnRNP L) as potential TAAs and immunotherapeutic targets is suggested by our results. Performance of their diagnostic value in a validation independent group will establish the clinical utility for early diagnosis of CaP. Additionally, analysis of the overexpression in tumors will help to define the role of these proteins in tumor growth and progression. Overall, our results

suggest that hypoxia modifies the cellular properties of CaP cells towards a phenotype that is more similar to tumor cells *in situ*. Introduction of pO_2 as a variable can constitute a tool for the development of more effective immunotherapy for CaP.

So what: The role of pO_2 in tumor biology has been unappreciated. Recently, tumor–associated hypoxia has been associated with malignant progression, metastasis, resistance to therapy, and poor clinical outcome. Our results validate the relevance of tumor-associated hypoxia in CaP and, more importantly, define the potential of hypoxia as a tool in the development of cellular vaccines for CaP.
We identified hypoxia-controlled genes with potential as prognostic factors in CaP. Validation of these genes anticipates applications in the clinic and research laboratory. Potential applications include refinement of the current prognostic tools for CaP and better tools to predict therapeutic outcome. In addition, a test for assessing hypoxia in tumors *in situ* could alleviate the problems of measuring pO_2 in tumor tissues. Because we found that molecular signature of tumor tissue under hypoxic conditions is retained after resection, the correlation of pO_2 and expression of hypoxia-controlled genes *in situ* could provide a surrogate method to assess pO_2 in CaP tissue. Finally, our evidence of substantial sensitivity of CaP cells to hypoxia might lead to enhanced efficacy of therapy not only for CaP, but also serve as a paradigm for other forms of cancer.

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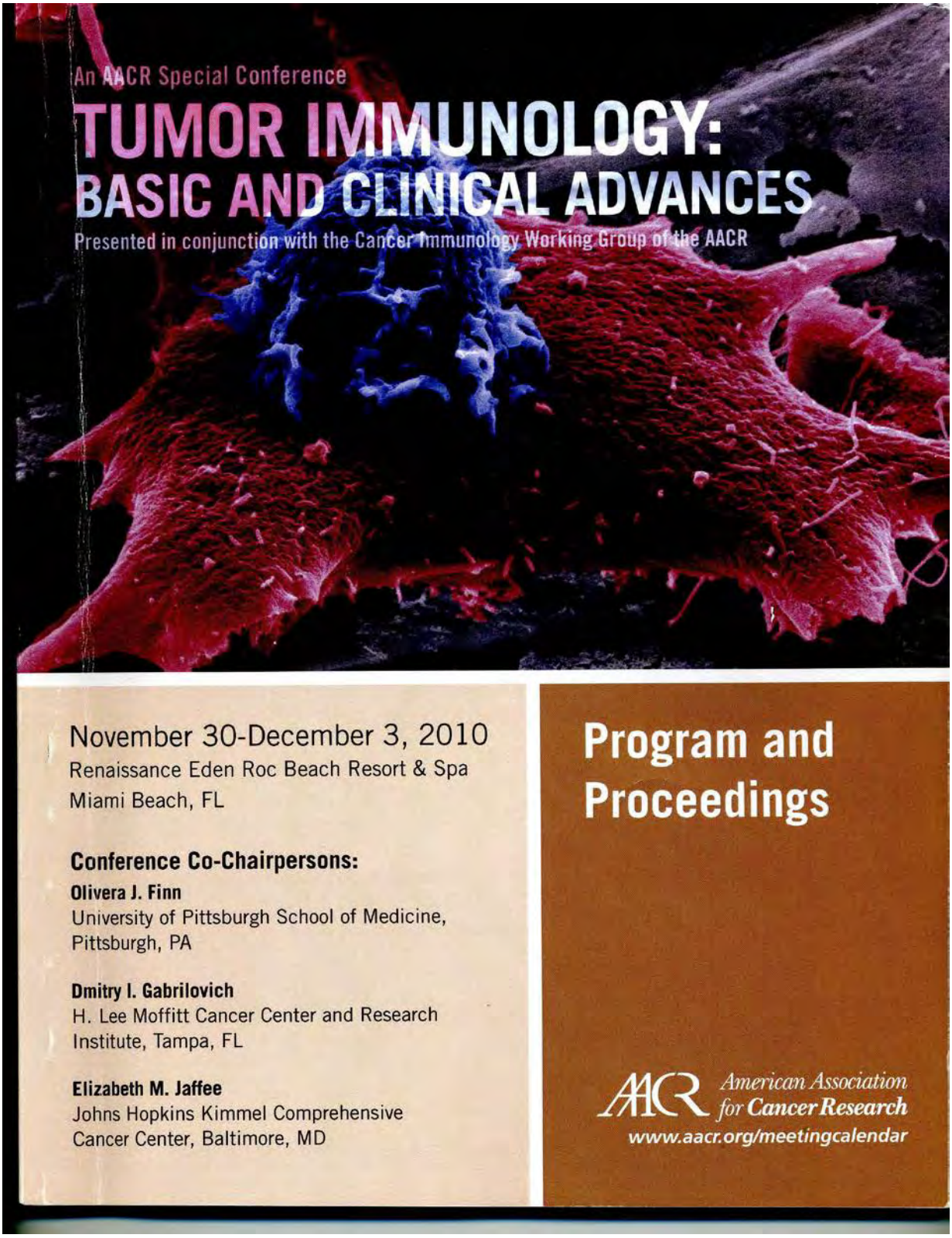
Appendices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xenografts (volume 100mm^3). Weekly systemic administration of MRG partially yet significantly inhibited tumor growth, as compared to injection of carrier only. MRG1 did not stimulate Antibody Dependent Cellular Cytotoxicity mediated elimination of melanoma cells in SCID-NOD mice. Single intravenous injection of melanoma specific human T cells yielded partial inhibition of tumor growth. Importantly, the treatment with a single adoptive cell transfer clearly synergized with weekly MRG1 therapy (>75% inhibition).
In conclusion, we show that CEACAM1 directly enhances various aggressive features of melanoma and provisionally position MRG1 as a potential novel anti-melanoma drug. Importantly, 75% of clinical melanoma specimens express CEACAM1 and are thus suitable for CEACAM1-targeted therapy. This represents a novel modular line of therapy that can stand alone and also potentially synergize with other therapeutic modalities.

A3 Hypoxic exposure as a novel strategy to identify tumor-associated antigens in prostate cancer. Christian R. Gomez, Farhad Kosari, Claire A. Schreiber, Gaylord J. Knutson, George Vasmatzis, Stanimir Vuk-Pavlovic. Mayo Clinic, Rochester, MN.

Hypoxia is a hallmark of the environment of many tumors. We hypothesize that it can modulate expression of tumor-associated antigens (TAAs) and thus affect immunity-based therapeutic strategies. To test the effects of hypoxia on prostate cancer (CaP), we studied global gene expression in 100 CaP tissues and 71 samples of adjacent benign tissues. RNA was extracted from cancer cells isolated by laser-capture microdissection (LCM) or without isolation ("bulk tissue"). We identified 24 hypoxia-associated genes significantly overexpressed in CaP ($p \leq 0.02$), both bulk tissue and LCM. Among these genes, cyclin B1 [CCNB1], disc large (drosophila) homolog-associated protein 5 [DLG7], and hyaluronan-mediated motility receptor [HMMR] were associated with Gleason score and with disease prognosis. Since the products of CCNB1 and HMMR genes have been recently identified as TAAs, our results suggest the potential of DLG7 and other candidate genes as possible TAAs. In addition, we propagated CaP cell lines LnCaP, VCaP and DU145 in hypoxia (pO2=2 kPa) and compared them with normoxically grown cells (pO2=20 kPa). Hypoxic cells proliferated faster and secreted more vascular endothelial growth factor (VEGF; $p<0.05$). As expected, transcription profiles revealed differential gene expression in cells grown in hypoxia relative to those in normoxia. Interestingly, transcripts for pyruvate dehydrogenase kinase isozyme 1 (PDK1), nuclear prelamin A recognition factor (NARF), glucose phosphate isomerase (GPI), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] were overexpressed in hypoxia in all three cell lines ($p<0.05$) both in bulk tissue and LCM isolated cells ($p<0.005$). Also, we observed a 30–60 percent hypoxia-associated increase in CCNB1 and DLG7 expression in VCaP cell line, the only one harboring the TMPRSS2-ERG fusion (present in 40–60 percent of CaP patients) among the three cell lines studied. To determine if hypoxia affects immunogenicity of CaP cells, we screened the sera from CaP patients (n=20) and healthy controls (n=13) for spontaneous antibodies cross–reactive with spots resolved on 2D electropherograms of lysates of LnCaP cells grown in hypoxia and normoxia. CaP patient sera bound to numerous spots in both electropherograms, but the binding patterns differed significantly. Overall, our data indicate that hypoxia affects gene expression and antigenic signature in CaP. Our ongoing experiments will resolve the question whether CaP cells grown in hypoxia are more akin to of tumor cells in situ than are cells grown in normoxia and will further identify and validate selected candidate TAAs in CaP patients.
Support: DOD PC094680 (CRG), Minnesota Partnership for Biotechnology and Medical Genomics, Mayo Clinic Prostate SPORE 5P50CA091956 (FK, SV-P), Adelyn Luther, Singer Island, Florida (SV-P); and Mayo Clinic Cancer Center (SV-P).

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Presentation Abstract

Abstract Number:	LB-308
Presentation Title:	Hypoxia affects gene expression and proteome of prostate cancer cells
Location:	Exhibit Hall A4-C, Poster Section 38
Author Block:	*Christian R. Gomez, Gaylord J. Knutson, Claire A. Schreiber, Farhad Kosari, George Vasmatzis, Stanimir Vuk-Pavlovic.* Mayo Clinic College of Medicine, Rochester, MN
Abstract Body:	Tumor-associated hypoxia facilitates malignant progression, metastasis and poor prognosis in prostate cancer (CaP), however, effects of oxygen tension (pO_2) in CaP are just beginning to be investigated. To establish mechanisms whereby hypoxia enhances malignant properties and survival of CaP and to identify pO_2-regulated tumor-associated macromolecules, we propagated CaP cell lines LnCaP, VCaP and DU145 in hypoxia (pO_2=2 kPa) and compared them with normoxically grown cells (pO_2=20 kPa); hypoxic cells proliferated faster and secreted more vascular endothelial growth factor (p<0.05). Transcriptome studies revealed different gene expression in cells grown in hypoxia relative to those in normoxia. Interestingly, in hypoxic cells transcripts associated with cancer and urologic disease were overexpressed in comparison to normoxic cells (p<0.001) suggesting an association of low pO_2 and aggressive features of CaP. Further, we analyzed the transcriptome in primary CaP cells isolated by laser-capture microdissection (LCM) and whole CaP tissue. We compared the data to those from CaP cells cultured at pO_2=20 kPa or pO_2=2 kPa. Notably, hypoxia increased transcript levels for pyruvate dehydrogenase kinase isozyme 1, nuclear prelamin A recognition factor, glucose phosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase in all three cell lines (p<0.05) to levels comparable to those found in primary bulk tissue and LCM isolated cells (p<0.005). This finding suggests that gene expression in hypoxically cultured cells is more akin to that in tumor cells *in situ* than are cells grown normoxically. By 2D-gel electrophoresis, we found that change in pO_2 affected the proteome mostly quantitatively (*i.e.*, by change in spot intensity). Our results suggest that hypoxia affects transcriptome and proteome in CaP cells. In addition, we screened 20 patient sera and 20 healthy control sera for spontaneous antibodies cross-reactive with VCaP cells and found that the sera reacted with numerous proteins, some previously reported to elicit an immune response in CaP patients (*e.g.*, nucleoporin 62 and transitional endoplasmic reticulum ATPase). By this method we detected numerous novel autoantigens (under validation). Currently we are clarifying the pO_2 effects on the relationship of transcriptome, proteome and tumor-associated antigens in CaP cells. Support: DOD PC094680 (CRG), Minnesota Partnership for Biotechnology and Medical Genomics, Mayo Clinic Prostate SPORE 5P50CA091956 (FK), Mrs. Adelyn L. Luther, Singer Island, Florida and Mayo Clinic Cancer Center (SV-P).

American Association for Cancer Research
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Gomez, Christian R., Ph.D.

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Sent: Monday, May 02, 2011 9:51 AM
To: Gomez, Christian R., Ph.D.
Subject: FW: 7th ISABS Conference info - abstract accepted for poster presentation

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JUNE 20-24, 2011.
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Dear Prof. Stanimir Vuk-Pavlović,

On behalf of the Program Committee of the "**7th ISABS Conference in Forensic, Anthropologic and Medical Genetics and Mayo Clinic Lectures in Translational Medicine**", June 20-24, 2011, Bluesun hotel ELAPHUSA, Bol, Island of Brač, Croatia, we are pleased to inform you that the abstract entitled:

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Vedrana Skaro, Ph.D.
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6/6/2011

PROGNOSTIC VALUE OF HYPOXIA-CONTROLLED GENES IN HIGH-RISK PROSTATE CANCER

Christian R. Gomez, Jan Marie Munz, Farhad Kosari, R. Jeffrey Karnes, John C. Cheville, Cristiane M. Ida, Thomas J. Sebo, Asha A. Nair, Hui Tang, George Vasmatzis, Stanimir Vuk-Pavlović. Mayo Clinic, Rochester, MN, USA.

Tumor–associated hypoxia has been associated with malignant progression, metastasis, resistance to therapy, and poor clinical outcome in prostate cancer (CaP). Prompted by the evidence that hypoxia affects CaP, we studied global gene expression in 100 CaP tissues and 71 samples of adjacent benign tissues. RNA was extracted from cancer cells isolated by laser-capture microdissection (LCM) or without isolation ("bulk tissue"). We found 24 hypoxia-associated genes significantly overexpressed in CaP ($p \leq 0.02$), both in bulk tissue and LCM. Among hypoxia-associated genes, the disc large (drosophila) homolog-associated protein 5 (DLG7), hyaluronan-mediated motility receptor (HMMR) and cyclin B1 (CCNB1) were associated with Gleason score and systemic progression. Since the products of HMMR and CCNB1 have been recently identified as molecular markers of CaP progression, we postulated that DLG7 has prognostic value also. To test this hypothesis we measured transcript levels for DLG7 in a 150-pair case-control cohort. The cases (progression to systemic disease within five years of surgery) and controls (no progression within seven years) were matched for clinical and pathologic prognostic variables, including grade, stage, and preoperative serum levels of PSA. The overall prognostic ability of DLG7, as tested in receiver operating characteristic analysis was of 0.74 (95% CL, 0.68 to 0.8), independent of the for ERG status. Overall, our data indicate that DLG7, a hypoxia-controlled gene have a prognostic value in high-risk CaP. Introduction of oxygen tension as a variable may constitute a tool for the identification of novel biomarkers for CaP.

Support: PCF Creativity Award (CRG), DOD PC094680 (CRG), Minnesota Partnership for Biotechnology and Medical Genomics (GV), Mayo Clinic Prostate SPORE 5P50CA091956 (FK, SV-P), Adelyn Luther, Singer Island, Florida (SV-P); and Mayo Clinic Cancer Center (SV-P).

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Targeting hypoxia for more effective immunotherapy of prostate cancer

Short Title:
hypoxia and immunotherapy of CaP

Author Block: Christian R. Gomez[1], Claire A. Schreiber[2], Gaylord J. Knutson[2], Cristine Charlesworth[2], Stanimir Vuk-Pavlovic[2]. [1]University of Mississippi Medical Center, Jackson, MS; [2]Mayo Clinic, Rochester, MN

Abstract:
In early clinical trials, vaccination with allogeneic cancer cells enhanced the overall survival in some patients with malignancies. Because the administered vaccine cells were cultured at pO_2=20 kPa, it is unclear whether they provided an adequate antigen match to tumor cells *in situ* where pO_2 is generally much lower. Thus, we postulate that hypoxically grown vaccine cells will provide a better antigen match to tumors *in situ*. We are testing this hypothesis by studying the effects of hypoxia on prostate cancer (CaP) cells. LnCaP and VCaP cells were cultured at pO_2=2 kPa or 20 kPa and analyzed by 2-D gel electrophoresis. The difference in pO_2 affected the proteome mostly quantitatively (*i.e.*, by change in spot intensity). Using a threshold of fivefold change, we found that hypoxia decreased the levels of thirteen proteins and increased of four in VCaP cells. These results are in line with the reports showing that hypoxia affects expression only of a small fraction of total cellular protein and that the content of total protein is not altered significantly. To determine if the hypoxia-dependent changes affected immune reactivity of the cells, we took advantage of the spontaneous autoantibodies against tumor-associated antigens (TAAs). Hence, we compared the binding to lysates of LnCaP and VCaP cells of antibodies pooled from CaP patients (n=25), healthy controls (n=25), rheumatoid arthritis (n=17), colorectal cancer (n=10) and lung cancer (n=10). CaP patient sera plasma reacted with numerous spots, some even in control groups and thus considered nonspecific. CaP sera plasma specifically bound to seven spots; four were hypoxia-specific. All selected spots were excised from the gel, trypsin-digested, and identified by MALDI-TOF mass spectrometry as heat shock 70 kDa protein 4; protein disulfide isomerase A3; heterogeneous nuclear ribonucleoprotein L and leucine-rich repeat-containing protein 47. With the exception of the latter, the proteins have been identified or validated as TAAs before, but apparently none in CaP. CaP patient antibodies bound to lysates of LnCaP cells grown at pO_2=2 kPa , but less than to such VCaP cell lysates. Our results confirmed the findings that hypoxia affects the proteome mostly quantitatively. Lysates of hypoxic CaP cells revealed novel potential TAAs suggesting the potential immunologic relevance of antigens in the hypoxic proteome. Thus, pO_2 control can provide a tool for the development of more effective immunotherapy for CaP and possibly other cancers.
Present address CRG: University of Mississippi Cancer Institute, Jackson, MS, USA. Support: DOD PC094680 (CRG), PCF Creativity Award (CRG), Mayo Clinic Prostate SPORE 5P50CA091956 (SV-P); Mrs. Adelyn L. Luther, Singer Island, Florida and Mayo Clinic Cancer Center (SV-P).

Category (Complete): Tumor immunology targets
Keywords/Indexing (Complete): Tumor hypoxia ; Immunotherapy ; Prostate cancer ; Tumor antigen

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(220319_1) - Prognostic value of hypoxia-associated genes in prostate cancer

Christian R. Gomez[1], Farhad Kosari[2], Jan Marie Munz[2], Claire Schreiber[2], Gaylord Knutson[2], Cristine Charlesworth[2], R. Jeffrey Karnes[2], John Cheville[2], George Vasmatzis[2], Stanimir Vuk-Pavlovic[2].

[1]University of Mississippi Medical Center, Jackson, MS, [2]Mayo Clinic, Rochester, MN.

Tumor-associated hypoxia has been associated with malignant progression, metastasis and resistance to therapy. We studied the role of oxygen tension (pO2) in modulating the properties of cultured human prostate cancer (CaP) cells. In air, where oxygen tension (pO2) is approximately 20 kPa, cultured CaP cells expressed lower levels of transcripts associated with cancer and urologic disease; reducing pO2 to 2 kPa made these levels comparable to those in surgical CaP specimens suggesting that hypoxically cultured cells, rather than normoxically cultured cells, express genes at levels akin to those in tumor cells in situ. In human primary CaP tissue, transcripts of hypoxia-associated genes DLG7, CCNB1 and HMMR were overexpressed and associated with Gleason score and disease prognosis; this implies the potential prognostic value of these transcripts. While hypoxia affected the CaP cell proteome mostly quantitatively, it did affect expression of tumor-associated antigens and suggested the potential therapeutic relevance of the hypoxic antigenic landscape. Overall, our results suggest that hypoxia modifies cellular properties of cultured CaP cells towards a phenotype more similar to tumor cells in situ. Introduction of pO2 as a variable can constitute a tool for the identification of more effective prognostic biomarkers and development of better therapy for CaP and, possibly, other tumors.

Category: Prognostic Signatures

Keywords: hypoxia; biomarkers; prognosis

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Targeting hypoxia for the identification of novel biomarkers for prostate cancer

Short Title:
Hypoxia biomarkers prostate cancer

Author Block: Christian R. Gomez[1], Jan Marie Munz[2], Farhad Kosari[2], Jeffrey Karnes[2], John Cheville[2], Cristiane Ida[2], Thomas Sebo[2], George Vasmatzis[2], Stanimir Vuk-Pavlovic[2]. [1]University of Mississippi Medical Center, Jackson, MS; [2]Mayo Clinic, Rochester, MN

Abstract:
Tumor-associated hypoxia has been associated with malignant progression, metastasis, resistance to therapy, and poor clinical outcome in prostate cancer (CaP). Prompted by the evidence that hypoxia affects CaP, we studied global gene expression in 100 CaP tissues and 71 samples of adjacent benign tissues. RNA was extracted from cancer cells isolated by laser-capture microdissection (LCM) or without isolation ("bulk tissue"). We found 24 hypoxia-associated genes significantly overexpressed in CaP ($p \leq 0.02$), both in bulk tissue and LCM. Among hypoxia-associated genes, the disc large (drosophila) homolog-associated protein 5 (DLG7), hyaluronan-mediated motility receptor (HMMR) and cyclin B1 (CCNB1) were associated with Gleason score and systemic progression. Since the products of HMMR and CCNB1 have been recently identified as molecular markers of CaP progression, we postulated that DLG7 has prognostic value also. To test this hypothesis we measured transcript levels for DLG7 in a 150-pair case-control cohort. The cases (progression to systemic disease within five years of surgery) and controls (no progression within seven years) were matched for clinical and pathologic prognostic variables, including grade, stage, and preoperative serum levels of PSA. The overall prognostic ability of DLG7, as tested in receiver operating characteristic analysis was of 0.74 (95% CL, 0.68 to 0.8), independent of the for ERG status. Overall, our data indicate that DLG7, a hypoxia-controlled gene have a prognostic value in high-risk CaP. To better define the value of DLG7 as a molecular predictor of CaP progression, we are currently assessing DLG7 protein levels by immunohistochemistry. Then we will analyze the association of DLG7 levels with clinical parameters. Introduction of oxygen tension as a variable may constitute a tool for the identification of novel biomarkers for CaP.
Present address CRG: University of Mississippi Cancer Institute, Jackson, MS, USA. Support: DOD PC094680 (CRG), PCF Creativity Award (CRG), Mayo Clinic Prostate SPORE 5P50CA091956 (SV-P); Mrs. Adelyn L. Luther, Singer Island, Florida and Mayo Clinic Cancer Center (SV-P).
.

Author Disclosure Information: C.R. Gomez: None. J. Munz: None. F. Kosari: None. J. Karnes: None. J. Cheville: None. C. Ida: None. T. Sebo: None. G. Vasmatzis: None. S. Vuk-Pavlovic: None.
Sponsor (Complete):
Category and Subclass (Complete): MCB03-01 Molecular marker studies
Keywords/Indexing (Complete): Hypoxia ; Biomarker ; Prostate cancer
Research Type (Complete): Translational research
Submission Details (Complete):
*Primary Organ Site: Genitourinary cancers: prostate
*Special Consideration: Not Applicable
*Choose Chemical Structure Disclosure: NOT APPLICABLE. No compounds with defined chemical structures were used.
*Please explain (maximum 250 characters with spaces): : NA

Financial Support for Attendance (Complete):
Payment (Complete): Your credit card order has been processed on Monday 24 October 2011 at 1:24 PM.
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Hypoxia-Reactive Proteins as Potential Tumor-Associated Antigens in Prostate Cancer

Tangeng Ma[1], Abdelouahid Elkhattouti[1], Farhad Kosari[2], R. Jeffrey Karnes[2], John C. Cheville[2], George Vasmatzis[2], Stanimir Vuk-Pavlović[2], Christian R. Gomez[1,2]. [1]University of Mississippi Medical Center, Jackson, MS; [2]Mayo Clinic, Rochester, MN.

Tumor–associated hypoxia has been linked to malignant progression, metastasis and resistance to therapy. We studied the role of oxygen tension (pO_2) in modulating the proteome and immunogenicity of cultured human prostate cancer (CaP) VCaP and LnCaP cells. Hypoxia affected the CaP cell proteome mostly quantitatively; and particularly it did affect expression of tumor–associated antigens (TAAs). Three potential TAAs; two hypoxia-specific (HSP70 and hnRNP L) and one hypoxia-nonspecific (HSP60) were selected for validation. Independently of the cell line used as antigens source, the frequency of HSP60 autoantibodies was elevated in the CaP patient plasma (13.0%) relative to healthy controls (1.3%); colorectal cancer (7.14%); lung cancer (10.0%); renal cell carcinoma (5.0%); and rheumatoid arthritis (0%). The frequency of HSP70 autoantibodies was not elevated in CaP patients (5.3%) when compared to healthy controls (3.7%). Even more, higher autoantibodies frequencies were found in other tumors (e.g. 20.0% in lung tumor) and in rheumatoid arthritis (17.65%). These data suggest a specific humoral response against HSP60 in CaP. Further evaluation in an independent validation group is needed to evaluate the performance and diagnostic value of HSP60 autoantibodies. In additional experiments we evaluated protein expression for hnRNP L, detected by Western Blot technique, in CaP tissue. The level of hnRNP L protein was significantly higher ($p<0.05$); with a 2.04 fold increase compared to control prostate tissue. Similar results (4.89-fold higher expression relative to control tissue; $p<0.005$) were found for HURP protein expression, a hypoxia-controlled gene recently validated in our lab as a prognostic value in high-risk CaP. Currently, we are performing the histopathological evaluation of those candidates in CaP tissue. Our results suggest the value of selected candidates (i.e. HSP60 and hnRNP L) as potential TAAs and immunotherapeutic targets for CaP. Additionally, their overexpression in tumors suggests their role in tumor growth and progression. Introduction of pO_2 as a variable can constitute a tool for the identification of more effective prognostic biomarkers and development of better therapy for CaP and, possibly, other tumors.

Funding: PCF Creativity Award (CRG), DOD PC094680 (CRG), Minnesota Partnership for Biotechnology and Medical Genomics (GV), Mayo Clinic Prostate SPORE 5P50CA091956 (FK, SV-P), Adelyn Luther, Singer Island, Florida (SV-P); and Mayo Clinic Cancer Center (SV-P).

Print this Page

AACR American Association for Cancer Research | ANNUAL MEETING 2013
April 6-10, 2013, Walter E. Washington Convention Center, Washington, DC

Presentation Abstract

Abstract Number:	4056
Presentation Title:	**Hypoxia as a tool for identifying prostate cancer-associated antigens**
Presentation Time:	Tuesday, Apr 09, 2013, 1:00 PM - 5:00 PM
Location:	Hall A-C, Poster Section 25
Poster Board Number:	21
Author Block:	Tangeng Ma[1], Abdelouahid Elkhattouti[1], Farhad Kosari[2], R. Jeffrey Karnes[2], John C. Cheville[2], George Vasmatzis[2], Stanimir Vuk-Pavlović[2], Christian R. Gomez[1]. [1]University of Mississippi Medical Center, Jackson, MS; [2]Mayo Clinic, Rochester, MN
Abstract Body:	So far, therapeutic vaccination with allogeneic cancer cells has been only marginally effective. A reason for failure could stem from the fact that vaccine cells are usually cultured in air (pO_2=20 kPa), while tumors in situ are often highly hypoxic. Hence, it is possible that vaccine cells expressed antigens differently than tumor cells in situ. We postulate that cells grown at low pO_2 provide a better antigen match to tumors in situ and could be more effective vaccines. We tested the former hypothesis by comparing prostate cancer (CaP) cells propagated at pO_2 = 2 kPa and 20 kPa. To identify potential tumor-associated antigens, we prepared CaP cell lysates, resolved them by 2D electrophoresis and immunoblotting using spontaneous antibodies from plasma derived from CaP patients and control subjects. Antibody-labeled spots were analyzed by MALDI-TOF mass spectrometry. Among identified molecules, we selected hypoxia-regulated HSP70 and hnRNP L and hypoxia-independent HSP60 and determined by ELISA the frequency of plasma samples reacting with these molecules two standard deviations above their reaction with normal sera. Frequency of HSP60-reactive plasma was 1/76 in healthy controls (HC; 1.3%), while it was 7/54 in CaP patients (13.0%; $p<0.05$). The corresponding values were 1/14 in colorectal cancer (7.1%), 1/10 in lung cancer (LC; 10.0%), 1/20 in renal cell carcinoma (5.0%) and 0/17 in rheumatoid arthritis (RA; 0%), not different from healthy controls. These data suggest that CaP patients develop humoral immunity to HSP60. Levels of autoantibodies to HSP70 in CaP patients (2/38, 5.3%) did not differ from HC (2/54, 3.7%) while they did differ from HC in LC (2/10, 20.0%) and RA (3/17, 17.7%). Similarly, frequency of autoantibodies to hnRNP L did no differ between HC (3/49, 6.1%) and CaP patients (2/38, 5.3%). Further, we evaluated the expression of hnRNP L protein in native CaP tissue by Western blot. In contrast to the levels of autoantibodies in plasma, hnRNP L antigen was present at twice the levels in CaP tissue (N=8) than in control benign prostate tissue (N=4; $p<0.05$). Overall, our results suggest that it is worthwhile to pursue evaluating of HSP60-specific autoantibodies as a CaP-associated marker. Currently, we are analyzing HSP60 and hnRNP L (hypoxia-regulated) protein and transcript expression by immunohistochemistry and PCR, respectively, in a large patient cohort. This approach could validate the use of hypoxia-sensitive molecules as effective prognostic biomarkers and help develop better therapy for CaP and, possibly, other tumors. Support: DOD PC094680 and PCF Creativity Award (both to CRG).

American Association for Cancer Research
615 Chestnut St. 17th Floor
Philadelphia, PA 19106

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Activity: Abstracts
Current Date/Time: 2/14/2013 11:56:08 AM

Identification and validation of hypoxia-specific tumor associated antigens in prostate cancer

Author Block: T. Ma[1], A. Elkhattouti[1], S. Vuk-Pavlović[2], C. R. Gomez[1];
[1]University of Mississippi Medical Center, Jackson, MS, United States, [2]Mayo Clinic, Rochester, MN, United States.

Abstract:
Prostate cancer (CaP) is characterized by unique prostate–associated antigens; hence, it has been considered a prime candidate for immunotherapy. Despite numerous laboratory advances, clinical outcomes have been partial and transient. The overall goal of this study is to optimize the effectiveness of therapeutic whole–cell CaP vaccines by taking into consideration tumor–associated hypoxia as a relevant determinant of tumor antigenicity. We propagated CaP cells at pO_2 = 2 kPa and 20 kPa. To identify potential tumor-associated antigens (TAAs), we prepared CaP cell lysates, resolved them by 2D electrophoresis and immunoblotting using spontaneous antibodies from plasma derived from CaP patients and control subjects. Antibody-labeled spots were analyzed by MALDI-TOF mass spectrometry and validated by ELISA. Three potential TAAs; two hypoxia-specific (HSP70 and hnRNP L) and one hypoxia-nonspecific (HSP60) were selected for validation. The frequency of HSP60 autoantibodies was elevated in the CaP patient plasma; however the frequency of HSP70 autoantibodies was not elevated in CaP patients relative to healthy controls; even more, higher autoantibodies frequencies were found in other tumors and in rheumatoid arthritis. Protein expression for hnRNP L (detected by Western Blot technique) was elevated in CaP tissue. Our results suggest the value of selected candidates (i.e. HSP60 and hnRNP L) as potential TAAs and immunotherapeutic targets for CaP. Additionally, their overexpression in tumors suggests their role in tumor growth and progression. Hypoxia may be used as a tool in the development of cellular vaccines and to identify novel diagnostic and prognostic tools for CaP.
CV:

Author Disclosure Information: C.R. Gomez: None.
Keyword (Complete): tumor associated antigens ; prostate cancer ; hypoxia ; vaccines
Topic (Complete): 6.6 Cancer immunotherapy and anti-tumor vaccines ; 6.7 Cell-based therapy ; 6.16 Biomarkers and clinical profiling of human immune responses
Presentation Preference (Complete): Oral preferred
Bursaries/Travel Grants (Complete):
I apply for the ICI 2013 BURSARY TO YOUNG IMMUNOLOGISTS (required): No
I apply for the IUIS/GATES FOUNDATION Travel Grant (required): No

Submission Fee (Complete):
I reside in an OECD country: Yes
I confirm that the information regarding my residency is accurate and that I have completed the payment if relevant. (required) : True

Oxygen Tension as a Modifier Agent of Prostate Cancer Cells: Potential for the Identification of Novel Tumor Microenvironment-relevant Imaging Probes.

Christian R. Gomez[1], Farhad Kosari[2], George Vasmatzis[2], Tangeng Ma[1], Stanimir Vuk-Pavlović[2] and Srinivasan Vijayakumar[1]

1 University of Mississippi Medical Center, Jackson, MS
2 Mayo Clinic Cancer Center, Rochester, MN, USA

Prolonged low oxygen tension (pO_2) in normal tissues results in injury and cell death, yet in tumors is a survival factor. We studied the effect of pO_2 in the malignancy of cancer cells. Prostate cancer (CaP) cells were grown in hypoxia at pO_2=2 KPa and in pO_2=20 KPa. At pO_2=2 kPa, a dynamic proliferation rate was observed in PCa cells (LnCaP, VCaP, DU145). For all these cell types pO_2=2 KPa reduced the proliferation rate by twofold ($p<0.05$). Among the reported effects of low pO_2 on promoting cancer progression there is stimulation in the production of vascular endothelial growth factor (VEGF), which mediates cancer growth and neoangiogenesis. When the effect of pO_2 on VEGF production was evaluated, pO_2=2 KPa resulted in approximately twofold increase in VEGF, relative to pO_2=20 KPa ($p<0.05$). These results suggest that PCa cells, exhibit plasticity to changing pO_2, thus reducing their proliferation rate and increasing their ability to secrete VEGF when in they are exposed to lower pO_2. Next, we isolated total RNA from LnCaP, VCaP, and DU-145 cultured at pO_2= 2 kPa or 20 kPa and assessed the transcriptome by Affymetrix Human U133 Plus 2.0 array. Genes expressed differently between experimental groups were identified, and Ingenuity Pathway Analysis was used to relate functions, pathways, networks, and unique features to genes differently expressed between two pO_2s. Interestingly, regardless of cell-specific changes in gene expression profiles, hypoxia-modified genes in molecular pathways associated with cancer and urologic diseases were overexpressed in comparison to normoxic cells ($p<0.001$) in all cell lines. These data suggest an association of low pO_2 and aggressive features of CaP. To test this hypothesis we compared the analyzed transcriptomes in CaP cells with those of CaP resected tissues. Notably, hypoxia increased transcript levels for pyruvate dehydrogenase kinase isozyme 1 (PDK1), nuclear prelamin A recognition factor (NARF), glucose phosphate isomerase (GPI), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in all cell lines ($p<0.05$) to levels comparable to those found in primary bulk tissue and LCM isolated cells ($p<0.005$). This finding suggests that gene expression in hypoxically cultured cells is more akin to that in tumor cells *in situ* than are cells grown normoxically. Particularly, expression of molecules critical for tumor cell metabolism— a well-recognized fact of interest for selection of tumor imaging probes; was elevated both in vitro and in CaP tumors. Overall, our results suggest that introduction of pO_2 is a tool for identifying novel tumor micro-environment targets relevant for treating, characterizing, and diagnosing cancer. Our future efforts include validation of the candidate probes in preclinical models and in materials from clinical trials.

Financial support: DOD PC094680 (CRG), PCF Creativity Award (CRG), Minnesota Partnership for Biotechnology and Medical Genomics (GV) and Mayo Clinic Prostate SPORE 5P50CA091956 (FK).

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Activity: Abstract Submission
Current Date/Time: 8/10/2013 12:58:39 PM

Role of Hepatoma Up-regulated Protein (HURP) in prostate cancer tumorigenesis

Short Title:
HURP in cancer tumorigenesis

Author Block: Abdelouahid El-Khattouti, Tangeng Ma, James R. Kent, Ingrid Espinoza, Christian R. Gomez
University of Mississippi Medical Center, Jackson, MS

Abstract:

Hepatoma Up-Regulated Protein (HURP) is a cell-cycle-regulated and microtubule-associated protein. It functions as a Ran GTPase effector and is involved in the stabilization of the mitotic kinetochore fibers. The expression of this protein is elevated in different tumor types and associated with tumor progression and aggressiveness. Also, a significant elevation of HURP expression was reported in normal stem cells including haematopoietic stem cells, mesenchymal stem cells, and mouse embryonic stem cells, but not in differentiated cells, an evidence for HURP as stemness maintenance factor. The aim of this study is to analyze the oncogenic potential of HURP in prostate cancer (PCa). We analyzed the expression level of HURP in PCa patients and found the transcripts of HURP were overexpressed in PCa. The elevated expression of HURP in PCa was found to be associated with higher Gleason score, systemic progression and poor prognosis. Furthermore, using a case-control cohort study we demonstrated the overall prognostic ability of HURP mRNA in advanced PCa, as tested in receiver operating characteristic analysis [AUC 0.74 (95% CL, 0.68 to 0.8)]. In addition, we analyzed the expression level of HURP protein in tumor tissue and in prostate benign and cancer cell lines; we found that the level of HURP protein was almost 5-fold higher ($p<0.005$) relative to control (cystoprostatectomy) tissue and in cancer cell lines compared to the benign cells. Overall, these data suggest a strong relationship between the elevated expression of HURP and the development and progression of PCa. Next, we set out a tetracyclin-regulated transient expression system model to study the effects of HURP overexpression in LNCaP cells. Data obtained from western blot revealed that HURP overexpression was associated with the phosphorylation of C-Jun-N-Terminal kinase (JNK) and the induction of both Fra1 and c-JUN proteins as well as with

the suppression of pro-apoptotic protein Bcl-Xs, and p53 without influencing the negative regulator MDM2. Also, the overexpression of HURP in CaP cell lines was found to enhance the expression of the anti-apoptotic protein Bcl-2 without influencing the anti-apoptotic protein. Conversely, viral titer of shHURP was found to inhibit the basal phosphorylation of JNK, to suppress the basal level of cyclin A, to increase the degradation of the inhibitor of NF-κB, to decrease the expression of PKC. Furthermore, down regulation of HURP was found to enhance the expression of p53 and to abolish its negative regulator MDM2, suggesting a central role for p53 in the modulation of HURP-induced cell proliferation and tumor progression. Also, the overexpression of HURP promoted the expression of the stemness markers Oct3/4 and Nanog, an evidence for the involvement of HURP in the maintenance of stemness properties in PCa cells. This idea is supported by the reported role of Nanog and Oct3/4 in promoting PCa stem cell characteristics as well as prostate resistance to androgen deprivation, and by our results showing decreased p53, with demonstrated roles in reprogramming, dedifferentiation, self-renewal, and pluripotency. Overall, our data suggest a potential role of HURP in PCa tumorigenesis. In addition, these findings suggest that HURP's stemness properties can contribute to its role in the etiology of PCa.

Support: DOD PC094680 (CRG), PCF Creativity Award (CRG).

Category (Complete): Biomarkers
Keywords/Indexing (Complete): Prostate cancer ; p53 ; Cell cycle ; Stem cells
Chemical Structure Disclosure (Complete):
Choose Chemical Structure Disclosure: NOT APPLICABLE. No compounds with defined chemical structures were used.
Please explain (maximum 250 characters with spaces): : NA

Financial Support for Attendance (Complete):
Additional Information (Complete):
Choose one of the following options: Yes, I allow this abstract to be available online approximately 2 weeks prior to the start of the conference.

Status: Complete

PROSTATE CANCER FOUNDATION

Howard R. Soule, PhD
Executive Vice President
Chief Science Officer

April 13, 2011

Christian Gomez, PhD
Biochemist
Mayo Clinic
200 First Street SW
Rochester, MN 55905

Dear Dr. Gomez:

It is our pleasure to inform you that you have successfully competed for a PCF Creativity Award titled, "*Hypoxia-Regulated DLG7 in Prostate Carcinogenesis and Prognosis*" at the Mayo Clinic. Your Creativity Award application was outstanding.

We are prepared to send a check for $150,000 for the first year of your award. Please be aware that this funding is in the form of an award and not a grant. As such, these funds must be sequestered from institutional overhead and other indirect costs. Our goal is to donate 100% of these funds directly to your research program.

PCF will issue payment upon receipt of the following information:

1. Complete and return institutional IRS form W-9.
2. Three recent photos of yourself with resolution >300 dpi. One should be a head shot and two should be "action" photos of you in the lab or clinic.
3. If this project employs human experimentation, we request proof that a successful IRB approval has been achieved.
4. If this proposal employs animal experimentation, we request proof of institutional approval for your investigation.
5. Please provide us with the award payment mailing address and name of person to whose attention the award check should be sent.

In addition, PCF requests the following:

1. Acknowledgement of PCF for any data presented or published that was funded by this award.
2. Attendance at the PCF Annual Scientific Retreat September 22-24, 2011, Incline Village, Nevada.

1250 Fourth Street, Santa Monica, CA 90401 | *Tel* 310.570.4596 *Fax* 310.570.4702 | *www.pcf.org*

We will provide a date for award activation when the above information is received and your check is sent. We will contact you to schedule a telephonic progress review by April 1, 2012. Measured productivity will ensure the second year of our commitment.

Best regards,

Howard R. Soule, PhD

HRS:ag

MAYO CLINIC

200 First Street SW
Rochester, Minnesota 55905
507-284-2511

November 22, 2011

SENT BY FEDERAL EXPRESS

Christian R. Gomez, Ph.D.
1840 Devine Street
Jackson, MS 39216

Dear Dr. Gomez:

I am pleased to offer you an appointment at Mayo Clinic as a research collaborator with Dr. Stanimir Vuk-Pavlovic, in the Division of Hematology for twelve months.

In keeping with research guidelines, the collaborator appointment is limited to no more than 90 days on campus during your appointment year. This appointment does not include any salary, honorarium, or benefits from Mayo Clinic.

As a research collaborator, you will be required to comply with Mayo Clinic policies during this appointment. You will not be eligible for patient or research subject contact, including clinical or surgical observation, remote access to the electronic medical record, or any other clinical applications.

Please arrange for health care insurance coverage for any hospitalization or physician services that may be required while at Mayo Clinic.

It is required that you read and complete the following forms and fax to (507) 538-0786 or e-mail as scanned documents to researchtp@mayo.edu:

- Appointment Information
- Confidential Information
- Intellectual Property
- Mayo Health Insurance Portability and Accountability (HIPAA) training
- Mayo Clinic Integrity Program - Patient Privacy & Security

If you have questions concerning your appointment, please let me know. I can be reached at golla.alice@mayo.edu or telephone at (507) 284-3281.

Sincerely,

Alice

Alice Golla, Staffing Specialist
Human Resources

AG:ljb

Enclosures
cc: Dr. Stanimir Vuk-Pavlovic

MAYO CLINIC

April 9, 2013

SENT BY E-MAIL TO: christian_gomez_usa@yahoo.com

Christian Gomez, Ph.D.
1840 Devine Street
Jackson, MS 39202
UNITED STATES

Dear Dr. Gomez:

I am pleased to offer you an appointment at Mayo Clinic as a research collaborator with Dr. Stanimir Vuk-Pavlovic in the Division of Hematology for one year beginning April 22, 2013.

In keeping with research guidelines, the collaborator appointment is limited to no more than 90 days on campus during your appointment year. This appointment does not include any salary, honorarium, or benefits from Mayo Clinic.

As a research collaborator, you will be required to comply with Mayo Clinic policies during this appointment. You will not be eligible for patient or research subject contact, including clinical or surgical observation, remote access to the electronic medical record, or any clinical applications.

Please arrange for health care insurance coverage for any hospitalization or physician services that may be required while at Mayo Clinic.

Satisfactory background, employment, and education checks
Please go to the Verified Credentials, Inc. (VCI) web site and complete the electronic VCI form within 48 hours of receiving this letter. The web site is www.myvci.com/rochestermayoresearch and it is recommended that you use Internet Explorer 7, Internet Explorer 8, Internet Explorer 9, or Firefox. Include educational degrees obtained post high school. You will not be able to attend orientation or begin your appointment until this requirement has been completed and approved.

It is required that you read and complete the following forms and fax to (507) 538-0786 or e-mail as scanned documents to researchtp@mayo.edu:

- Appointment Information
- Confidential Information
- Intellectual Property
- Mayo Health Insurance Portability and Accountability (HIPAA) training
- Mayo Clinic Integrity Program - Patient Privacy & Security

If you have questions concerning your appointment, please let me know. I can be reached at

golla.alice@mayo.edu or telephone at (507) 284-3281.

Sincerely,

Alice J. Golla

Alice J. Golla, Staffing Specialist
Human Resources

AJG/REQ#21922

Enclosures
cc: Dr. Stanimir Vuk-Pavlovic

THE UNIVERSITY OF MISSISSIPPI
MEDICAL CENTER

Office of the Vice Chancellor
for Health Affairs
2500 North State Street
Jackson, Mississippi 39216-4505
(601) 984-1010

September 28, 2011

Dr. Christian Gomez
Department of Pathology/Cancer Institute
UMMC – Campus Mail

Dear Dr. Gomez:

On behalf of the University of Mississippi Medical Center (UMMC), I want to personally congratulate you on your appointment as Associate Professor in the Department of Pathology in the School of Medicine.

I am certain your talents will enrich this department and contribute to the exciting direction in which our institution is moving.

Best wishes and I look forward to working with you in the future.

Sincerely,

James E. Keeton, M.D.
Vice Chancellor and Dean

JEK/pr

University of Mississippi
Health Care
University Hospitals & Health System

May 10, 2013

Christian Gomez, PhD
Department of Pathology
University of Mississippi Medical Center
2500 North State Street
Jackson, Mississippi 39216-4505

Dear Dr. Gomez:

Re: Secondary Appointment in Radiation Oncology

Congratulations, your secondary appointment as Associate Professor in the Department of Radiation Oncology has been approved effective November 8, 2012. We are happy to have you as an employee of our department.

If you need anything further, please do not hesitate to let me know.

Thank you again for your service to the University of Mississippi Medical Center and Radiation Oncology.

Kindest regards,

Hope K. Burton
Assistant to the Chairman
Business Operations Supervisor
Department of Radiation Oncology

Radiation Oncology • Jackson Medical Mall
350 W. Woodrow Wilson Drive • Suite 1600 • Jackson, Mississippi 39213
T 601.984.2550 • F 601.815.6876 • umhc.com

Clinical Programs of The University of Mississippi Medical Center

The University of Mississippi Medical Center
2500 North State Street
Jackson, Mississippi 39216-4505

School of Graduate Studies
in the Health Sciences

Phone (601) 984-1195
Fax (601) 815-9440

May 10, 2012

Christian René Gomez, PhD
Associate Professor, Department of Pathology
University of Mississippi Medical Center
2500 N. State Street
Jackson, MS 39216

Dr. Gomez:

You have been recommended for membership to the graduate faculty of the School of Graduate Studies in the Health Sciences. The purpose of this letter is to inform you that you have been accepted as a full member of the School of Graduate Studies' Graduate Faculty.

We look forward to your contributions to the graduate program.

Joey P. Granger

Joey P. Granger, Ph.D.
Dean, School of Graduate Studies

JPG/dm

cc: Julius M. Cruse, M.D., Ph.D.
Director, Graduate Studies in Pathology

DEPARTMENT OF VETERANS AFFAIRS
G. V. (Sonny) Montgomery
Medical Center
1500 East Woodrow Wilson
Jackson, MS 39216

May 29, 2013

586/05-J

Dr. Christian Gomez
1840 Devine Street
Jackson, MS 39202

Dear Dr. Gomez,

Welcome to the G.V. Sonny (Montgomery) Veterans Affairs Medical Center. You are appointed to our facility as a Without Compensation Employee, Associate Professor/Co-Investigator, from June 6, 2013 until September 30, 2013. You are authorized to perform services as directed by Dr. Chadwick Huckabay, during your period of affiliation with our facility.

Upon acceptance of this assignment, you will receive no monetary compensation and will not be entitled to benefits normally given to paid employees of the Department of Veterans Affairs, such as leave, retirement, etc. Cash is unauthorized in lieu of any of these benefits.

The G.V. Sonny (Montgomery) Veterans Affairs Medical Center has a no smoking policy which prohibits smoking within the facility. All employees are expected to adhere to this policy in order to create a smoke free environment.

If you agree to these conditions, please sign the statement below. This agreement may be terminated at any time by either party in writing of intent. If you have any questions, please contact Cassandra Evans, HR Assistant, at 601-362-4471 extension 4368.

Sincerely yours,

for

Michael Palmier
Acting Chief, Human Resources

Signature	847-769-1185 Local Telephone Number	06.06.13 Date

AMENDMENT OF SOLICITATION/MODIFICATION OF CONTRACT

1. CONTRACT ID CODE	PAGE OF PAGES
S	1 | 2

2. AMENDMENT/MODIFICATION NO.	3. EFFECTIVE DATE	4. REQUISITION/PURCHASE REQ. NO.	5. PROJECT NO. (If applicable)
P00002	21-May-2012	W91ZSQ9312N661	

6. ISSUED BY	CODE	7. ADMINISTERED BY (If other than item 6)	CODE
USA MED RESEARCH ACQ ACTIVITY 820 CHANDLER ST FORT DETRICK MD 21702-5014	W81XWH	US ARMY MEDICAL RESEARCH ACQUISITION ACT ATTN: AMBER STILLRICH AMBER.STILLRICH@AMEDD.ARMY.MIL 820 CHANDLER STREET FORT DETRICK MD 21702	W81XWH

8. NAME AND ADDRESS OF CONTRACTOR (No., Street, County, State and Zip Code)		
UNIVERISTY OF MISSISSIPPI MEDICAL CENTER 2500 N STATE ST JACKSON MS 39216-4500		9A. AMENDMENT OF SOLICITATION NO.
		9B. DATED (SEE ITEM 11)
	X	10A. MOD. OF CONTRACT/ORDER NO. W81XWH-10-1-0225
CODE 1B5T7 FACILITY CODE	X	10B. DATED (SEE ITEM 13) 15-May-2010

11. THIS ITEM ONLY APPLIES TO AMENDMENTS OF SOLICITATIONS

☐ The above numbered solicitation is amended as set forth in Item 14. The hour and date specified for receipt of Offer ☐ is extended, ☐ is not extended.

Offer must acknowledge receipt of this amendment prior to the hour and date specified in the solicitation or as amended by one of the following methods: (a) By completing Items 8 and 15, and returning _____ copies of the amendment; (b) By acknowledging receipt of this amendment on each copy of the offer submitted; or (c) By separate letter or telegram which includes a reference to the solicitation and amendment numbers. FAILURE OF YOUR ACKNOWLEDGMENT TO BE RECEIVED AT THE PLACE DESIGNATED FOR THE RECEIPT OF OFFERS PRIOR TO THE HOUR AND DATE SPECIFIED MAY RESULT IN REJECTION OF YOUR OFFER. If by virtue of this amendment you desire to change an offer already submitted, such change may be made by telegram or letter, provided each telegram or letter makes reference to the solicitation and this amendment, and is received prior to the opening hour and date specified.

12. ACCOUNTING AND APPROPRIATION DATA (If required)

13. THIS ITEM APPLIES ONLY TO MODIFICATIONS OF CONTRACTS/ORDERS. IT MODIFIES THE CONTRACT/ORDER NO. AS DESCRIBED IN ITEM 14.

☐ A. THIS CHANGE ORDER IS ISSUED PURSUANT TO: (Specify authority) THE CHANGES SET FORTH IN ITEM 14 ARE MADE IN THE CONTRACT ORDER NO. IN ITEM 10A.

☐ B. THE ABOVE NUMBERED CONTRACT/ORDER IS MODIFIED TO REFLECT THE ADMINISTRATIVE CHANGES (such as changes in paying office, appropriation date, etc.) SET FORTH IN ITEM 14, PURSUANT TO THE AUTHORITY OF FAR 43.103(B).

☐ C. THIS SUPPLEMENTAL AGREEMENT IS ENTERED INTO PURSUANT TO AUTHORITY OF:

X D. OTHER (Specify type of modification and authority)
IAW USAMRAA General Terms & Conditions and request dated 04/18/2012

E. IMPORTANT: Contractor ☒ is not, ☐ is required to sign this document and return _____ copies to the issuing office.

14. DESCRIPTION OF AMENDMENT/MODIFICATION (Organized by UCF section headings, including solicitation/contract subject matter where feasible.)
Modification Control Number: kmoore124239

1. The purpose of this modification is to grant a twelve month extension without funds at no additional cost to the government.
2. An annual technical report will be due on 14 June 2012. The final report will now be due 14 June 2013.

Please see Summary of Changes for details. All other terms and conditions remain unchanged.

Except as provided herein, all terms and conditions of the document referenced in Item 9A or 10A, as heretofore changed, remains unchanged and in full force and effect.

15A. NAME AND TITLE OF SIGNER (Type or print)	16A. NAME AND TITLE OF CONTRACTING OFFICER (Type or print)
	SUSAN DELLINGER / CONTRACTING OFFICER TEL: 301-619-2090 EMAIL: susan.dellinger@amedd.army.mil

15B. CONTRACTOR/OFFEROR	15C. DATE SIGNED	16B. UNITED STATES OF AMERICA	16C. DATE SIGNED
_____ (Signature of person authorized to sign)		BY Susan M. Dellinger (Signature of Contracting Officer)	21-May-2012

EXCEPTION TO SF 30
APPROVED BY OIRM 11-84

30-105-04

STANDARD FORM 30 (Rev. 10-83)
Prescribed by GSA
FAR (48 CFR) 53.243

SECTION SF 30 BLOCK 14 CONTINUATION PAGE

SUMMARY OF CHANGES

SECTION 00010 - SOLICITATION CONTRACT FORM

CLIN 0001

The CLIN extended description has changed from PERIOD OF PERFORMANCE: 15 May 2010 - 14 June 2012 (research ends 14 May 2012) to PERIOD OF PERFORMANCE: 15 May 2010 - 14 June 2013 (research ends 14 May 2013).

DELIVERIES AND PERFORMANCE

The following Delivery Schedule item for CLIN 0001 has been changed from:

DELIVERY DATE	QUANTITY	SHIP TO ADDRESS	UIC
POP 15-MAY-2010 TO 14-JUN-2012	N/A	USA MED RESEARCH MAT CMD 1077 PATCHEL STREET FORT DETRICK MD 21702 FOB: Destination	W91ZSQ

To:

DELIVERY DATE	QUANTITY	SHIP TO ADDRESS	UIC
POP 15-MAY-2010 TO 14-JUN-2013	N/A	USA MED RESEARCH MAT CMD 1077 PATCHEL STREET BLDG 1077 FORT DETRICK MD 21702 FOB: Destination	W91ZSQ

(End of Summary of Changes)

August 10, 2013

CURRICULUM VITAE

Name: Christian René Gomez Basaure Ph.D.

E-mail: crgomez@umc.edu

Web Page: http://cancerinstitute.umc.edu/profiles/gomez_christian.html

Present Position: Associate Professor, Department of Pathology
Associate Professor, Department of Radiation Oncology
Full member Cancer Institute
University of Mississippi Medical Center
2500 N. State St. Suite G657, Jackson, MS 39216
Office: 601-815-3060, Fax 601-815-6806

Education

1988 - 1995 B.S. and M.S. in Biochemistry, School of Chemical and Pharmaceutical Sciences, University of Chile

1997-2003 Ph.D. Biomedical Sciences, University of Chile, Faculty of Medicine Date of Ph.D. completion January 2004

Research Training

1992 - 1994 Undergraduate thesis: Involvement of the Sodium/ATPase pump in chronic renal failure, Advisor: Dr. Miriam Alvo, Department of Physiology, University of Chile School of Medicine, Santiago, Chile

1995 - 1997 Research assistant: Glucocorticoid receptors in the development of Rheumatoid Arthritis: Development of a rat model, Advisor: Dr. Annelise Goecke, Department of Physiology, University of Chile School of Medicine, Santiago, Chile

1998 - 2000 Research assistant: CAAT/enhancer-binding protein signaling during the acute phase response of aged Fisher 344 rats, Advisor: Dr. Robin Walter, Department of Cellular and Molecular Biology, University of Chile School of Medicine, Santiago, Chile

2000 - 2004 Doctoral dissertation: Macrophage inflammatory protein 1-alpha as a modulating factor of the acute phase response: extension to the inflammatory response in aged individuals, Advisor: Dr. Felipe Sierra, Department of Cellular and Molecular Biology, University of Chile School of Medicine, Santiago, Chile

2004 - 2008 Postdoctoral Fellow: Aging and inflammatory responses. Supervisor: Dr. Elizabeth J. Kovacs, Loyola University Chicago, Stritch School of Medicine, Department of Cell Biology, Neurobiology and Anatomy and Department of Surgery, Maywood, IL

International Courses

Natural Antibodies in the Maintenance of Tolerance to Self: Lessons from Physiology and Therapy, Program of Immunology, Faculty of Medicine, University of Chile, Santiago, Chile, 15-16 December 1998

International Symposium and Training Course: "Cellular Signaling From Plasma membrane to the Nucleus", Program of Cellular and Molecular Biology, Faculty of Medicine, University of Chile, Santiago, Chile, 12-23 July 1999

International Symposium and Training Course: "International Course on Techniques for the Study of Functional Genomics", Program of Cellular and Molecular Biology, Faculty of Medicine, University of Chile, Santiago, Chile, 19 June – 1 July 2000

Training Course: "Molecular Biology of Aging", Marine Biological Laboratory, Woods Hole, MA, USA, 31 July – 19 August 2006

Faculty Appointments

2004 - 2007 Research Associate, Loyola University Chicago, Stritch School of Medicine, Department of Cell Biology, Neurobiology and Anatomy, Maywood, IL

2007 - 2008 Research Associate, Loyola University Chicago, Stritch School of Medicine, Department of Surgery, Maywood, IL

2008 - 2011 Research Associate, Stem Cell Laboratory, Department of Oncology, Mayo Clinic Cancer Center, Rochester, MN

2009 - 2011 Assistant Professor of Biochemistry/Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN

2010 - 2011 Assistant Professor, Division of Preventive, Occupational, And Aerospace Medicine, Mayo Clinic, Rochester, MN

2011 - Date Associate Professor, Department of Pathology, University of Mississippi Medical Center, Jackson, MS

2011 - Date Full Member, University of Mississippi Cancer Center, University of Mississippi Medical Center, Jackson, MS

2011 - Date Research Collaborator, Mayo Clinic, Rochester, MN

2012 - Date Full Member, The School of Graduate Studies' Graduate Faculty, University of Mississippi Medical Center, Jackson, MS

2012 - Date Associate Professor, Department of Radiation Oncology, University of Mississippi Medical Center, Jackson, MS

2013 - Date Researcher, Department of Urology, G. V. (Sonny) Montgomery VA Medical Center, Jackson, MS

Professional Awards

1998-2002 Chilean National Council for Science and Technology (CONICYT) doctoral scholarship

2000 International Travel Award: "Identification of genes that are differentially expressed during the acute phase response of senescent animals", Lab. Dr. Christian Cell, Lankenau Institute for Medical Research, Thomas Jefferson University, Wynnewood, PA, USA

2002 International Travel Award: "Characterization of differential hepatic expression of the chemokines MIP-1α, in aged rats, injected with bacterial endotoxin (LPS)", Lab. Dr. Christian Sell and Lab. Dr. Vincent Cristofalo, Lankenau Institute for Medical Research, Thomas Jefferson University, Wynnewood, PA, USA

2002 International Travel Award: "Standardization of the measurement of tissue and circulating levels of cytokines during the acute phase response of aged rats", Lab. Dr. Elizabeth J. Kovacs, Stritch School of Medicine, Loyola University, Maywood, IL, USA

2001 Distinguish Award for the "Best dissertation project on Gerontological Studies" Interdisciplinary Program for Gerontological Studies, University of Chile, Santiago, Chile

2004 Travel award to attend the Annual Meeting of the Society for Leukocyte Biology, Toronto, Canada

2004 Doctoral Medal, University of Chile, Santiago, Chile

2005 Travel award to attend the Annual Meeting of the Society for Leukocyte Biology, Oxford, England

2006 Young Investigator Travel Award to attend the Twenty-Ninth Annual Conference on Shock, Broomfield, CO, USA

2006 Travel award to attend the Annual Meeting of the Society for Leukocyte Biology, San Antonio, TX, USA

2010 AACR Minority Scholar in Cancer Research Award to attend the AACR Special Conference, Tumor Immunology: Basic and Clinical Advances. Miami, FL

2011 Prostate Cancer Foundation. Treatment Sciences Creativity Awards 2011

Professional Society Membership and Activities

2004 - 2008 Society for Leukocyte Biology, Member

2004 - 2008 Shock Society, Member

2009 - Date American Association for Cancer Research (AACR), Associate Member

2009 - Date Mayo Clinic Alumni Association, Member

2009 - Date AACR, Minority Scholar in Cancer Research, Member

Media Related Quotes and Interviews

2005 "Healthy Aging", Interview, University of Santiago Radio. Santiago, Chile

Journal Review Activity

2010 - Date Ad Hoc Reviewer: Journal of Leukocyte Biology, American Journal of Physiology-Advances in Medical Education

2012 - Date Editorial Board of Conference Papers in Immunology

2013 - Date Editorial Board of Journal of Clinical & Anatomical Pathology

2013 - Date Editorial Board of JSM Microbiology

Grant Review Panels

2012 - Date Training Clinical and Experimental Therapeutics peer review panel of the 2012 Prostate Cancer Research Program for the Department of Defense Congressionally Directed Medical Research Programs.

2012 - Date University of Mississippi Medical Center Intramural Research Support Program (IRSP) peer review panel

Teaching Experience

1999, 2001 Teaching Assistant, Course of Cellular Biology for Kinesics therapy and Occupational therapy and Medical Technology (First year students), Faculty of Medicine, University of Chile, Santiago, Chile

2002-2003 Teaching Assistant, Seminars on Biotechnology for Medical technology students (second year students), Mention clinical bio-analysis, Hematology and Blood bank, Faculty of Medicine, University of Chile, Santiago, Chile

2002-2003 Teaching Assistant, Cellular Biology course for Biochemistry students (fourth year students), Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile

2003-2004 Teaching Assistant, Workshop for Integration of Basic Sciences for Medical students (first year students), Faculty of Health Sciences, University Diego Portales, Santiago, Chile

2003-2006 Teaching Assistant, Course Structure and Function I for Medical students (first year students), Faculty of Health Sciences, University Diego Portales, Santiago, Chile

2003 Teaching Assistant, Course of Cell Biology for Ph.D. students, Faculty of Medicine, University of Chile, Santiago, Chile

2003 Teaching Assistant, Course of Advanced Genetics for Ph.D. students, Faculty of Medicine, University of Chile, Santiago, Chile

2004 Teaching Assistant, course of Cell Biology for Nursery and Medical Technology students (first year students), Faculty of Health Sciences, University Diego Portales, Santiago, Chile

2004 Teaching Assistant, Seminars in Molecular Biology for Medical Technology (fourth year students), Mention clinical Bio-analysis, Hematology and Blood bank, Faculty of Medicine, University of Chile, Santiago, Chile

2006 Teaching assistant, Medical Histology, The Stritch School of Medicine, Loyola University Medical Center, Maywood, IL

2013 Lecturer, Molecular Oncology for Graduate Students, University of Mississippi Medical Center, School of Graduate Studies in the Health Sciences, Jackson, MS

2013 Lecturer, Cellular and Molecular Biochemistry for Graduate Students, University of Mississippi Medical Center, School of Graduate Studies in the Health Sciences, Jackson, MS

Research Supervision

2005 Co-mentor: Stephanie Hirano, M.D. Student

2005 Mentor: Ying Peng, Ph.D. Candidate
Christine Regnell, M.S. Candidate
Shirin Birjandi, Ph.D. Candidate

All the students were at Elizabeth J. Kovacs' Laboratory at The Burn and Shock Trauma Institute, Loyola University Medical Center, Maywood, IL

2006-8	Mentor: Freddy Bustos, Constanza Fernández, Ana María Duhalde, M.D. Students, Methodology in research rotation, Universidad Diego Portales, Santiago, Chile
2009	Co-Mentor: Freddy Bustos, M.D. Student, research rotation, Stem Cells Lab, Mayo Clinic Cancer Center, Rochester, MN
2010-2011	Claire A. Schreiber, Luther College, Decorah, IA Research Assistant, Stem Cells Lab, Mayo Clinic Cancer Center, Rochester, MN
2011	Lauren Ulbrich, St. Mary's University, Winona, MN Summer student, Stem Cells Lab, Mayo Clinic Cancer Center, Rochester, MN
2011-Date	Tangeng Ma, Research Scientist III, University of Mississippi, Cancer Institute, Jackson, MS
2011-2012	Elizabeth Tarsi, Research Scientist II, University of Mississippi, Cancer Institute, Jackson, MS
2012-Date	Abdelouahid Elkhattouti, Postdoctoral Fellow I, University of Mississippi, Cancer Institute, Jackson, MS
2012	Appifani Binion, Tougaloo College, Tougaloo, MS, summer student, University of Mississippi, Cancer Institute, Jackson, MS
2012-Date	Ahmed Ejaeidi M.D., Graduate student, Department of Pathology, University of Mississippi, Cancer Institute, Jackson, MS
2013	Logan Fair, Second Year Medical Student, University of Mississippi School of Medicine, summer rotation, Cancer Institute, University of Mississippi, Cancer Institute, Jackson, MS
2013	James Kent Jr, Mississippi University for Women- Columbus, MS summer rotation, Cancer Institute, Jackson, MS
2013-Date	Tara Craft, Research Scientist II, University of Mississippi, Cancer Institute, Jackson, MS

Research Grant Support

Ongoing:

Tittle: Enhancing therapeutic cellular prostate cancer vaccines (PC094680) (P.I.).
Time commitment: 50%
Supporting agency: Department of Defense. New Investigator Award
Performance period: 04/15/10 – 04/31/13
Level of funding (direct costs): $225,000
Project goals: The overall goal of the proposed studies is designed to optimize the effectiveness of therapeutic whole–cell CaP vaccines. We hypothesize that hypoxically cultured CaP cells are more similar in their antigen landscape to CaP cells in situ than are normoxically cultured CaP cells
Specific aims: 1) To identify oxygen–tension responsive genes and proteins in the cells comprising a clinical–grade CaP cellular vaccine. 2) To validate differentially expressed molecules in CaP tissue in association with tissue hypoxia
Key personnel receiving salary support from this project: 100% effort CR Gomez. 50% effort allied staff

Tittle: Hypoxia-regulated DLG7 in prostate cancer carcinogenesis and prognosis. (P.I.)

Time commitment: proposed 50%
Supporting agency: Prostate Cancer Foundation. Treatment Sciences Creativity Awards 2011
Performance period: 05/01/11 – 05/01/13
Level of funding: $300.000 (direct costs)
Project goals: We found the transcripts of the discs large homolog-associated protein 5 (DLG7), a hypoxia-regulated gene, overexpressed in human primary prostate cancer and human prostate cancer cell lines. The overall goal of the proposed studies is to validate the role of DLG7 role in tumor progression
Specific aims: 1) To measure the levels of DLG7 transcripts in resected CaP tissues and study the association with survival. 2) To overexpress DLG7 in prostate cells (normal epithelium and tumorigenic cells) and compare tumorigenesis in the context of hypoxia.
Key personnel receiving salary support from this project: 50% effort CR Gomez. 100% effort postdoctoral fellow

Tittle: Improving effectiveness of cancer immunotherapy of prostate cancer. (P.I.)
Supporting Institution: University of Mississippi Cancer Institute–Seeding/Start-up Grant, Jackson, MS
Performance period: 09/26/11 – 09/26/16
Project goals: To characterize the antigenic landscape of hypoxically cultured prostate cancers cells and compare it to that of normoxic CaP cells. Testing of the role of the hypoxia-controlled gene–DLG7–in tumor progression
Key personnel receiving salary support from this project: 100% effort technical personnel and one postdoctoral fellow

Completed:
Tittle: Hyperbaric oxygen as mobilizer of stem cells and progenitors in senescent mice (Stanimir Vuk-Pavlovic, P.I.). Co P.I.
Time commitment: 30%
Supporting agency: Mayo Clinic, Division of Preventive, Occupational and Aerospace Medicine Small Grant Awards
Performance period: 04/01/09 – 12/30/09
Level of funding: $17,500
Project goals: The effects of hyperbaric oxygen (HBO) on mobilization of hematopoietic and stem and progenitor cells (HSPCs) and mesenchymal stromal cells (MSCs) from bone marrow into circulation of old mice were explored.
Specific Aims: 1) To measure the effects of HBO in young and old mice by flow cytometry after labeling white blood cells with pertinent fluorescent immunoreagents for HSPCs and MSCs. 2) To measure the levels of selected circulating cytokines involved in HSPCs and MSCs mobilization.
Key personnel receiving salary support from this project: 3% effort CR Gomez

Tittle: Hyperbaric oxygen as mobilizer of stem cells and progenitors in senescent mice. Extension of funds for 2010 (Stanimir Vuk-Pavlovic, P.I.). Co P.I.
Time commitment: 30%
Supporting agency: Mayo Clinic, Division of Preventive, Occupational and Aerospace Medicine Small Grant Awards
Performance period: 04/01/10 – 12/31/10
Level of funding: $10,000
Project goals: The mechanisms of age-related impairment of mobilization of both hematopoietic stem and progenitor cells (HSPCs) and mesenchymal stromal cells (MSCs) from bone marrow by hyperbaric

oxygen (HBO) into circulation of old mice were studied. Specific Aims: 1) To analyze the effects of aging and HBO on the expression of SDF-1/CXCR4 system, the critical regulator of SPCs function and homing. 2) To analyze the effects of aging and HBO on the regulation of nitric oxide (•NO)–mediated mechanism of MSCs mobilization by HBO synthesis.
Key personnel receiving salary support from this project: 3% effort CR Gomez

Applied
Tittle: Hypoxia enhances prostate cancer radioresistance by promoting cancer cell stemness. P.I.
Funding agency: BD Biosciences Research Grant Program-Stem Cell Grant.
Time commitment: 5%. Performance period: 07/01/12 – 07/01/14. Level of funding: $10,000.
Project goal: test the hypothesis that a low oxygen environment (where radiation is less effective due to the lack of the oxygen effect) increases the fraction of cancer stem cells in the population giving thus rise to a more resistant tumor.
Key personnel receiving salary support from this project: 10% effort flow cytometry core personnel.

Title: Role of Hepatoma Up-regulated protein (HURP) in prostate cancer, (El Khattouti PI; Gomez: Mentor)
Funding agency: CDMRP-PCRP
Time commitments: 0%
Level of funding: $115,000
Name and address of the funding agency's procuring Contracting/Grants Officer: N.A.
Project goal: We found that HURP was significantly overexpressed in human primary prostate cancer (PCa) tissues and in PCa cell lines. When overexpressed in PCa cells; HURP induced PCa aggressiveness features. Consequently, we hypothesize that overexpression of HURP induces PCa tumorigenesis. Reported evidence indicates that HURP regulates the stemness phenotype in normal stem cells. These findings added to our results showing that HURP overexpression in PCa cells promotes expression of stemness markers; allow us to propose that the tumorigenic role of HURP is related in part to its ability to enhance the stem cells properties of PCa cells. Aim 1: To determine the HURP-mediated effects on PCa tumorigenesis. Aim 2: To study if the tumorigenic effects of HURP in CaP are mediated by HURP's stemness-enhancing properties
Key personnel receiving salary support from this project: 100% effort postdoctoral fellow.

Title: GAP1 Unique TMA project (Zhou PI; Gomez: Collaborator)
Supporting agency: Movember
Time commitments: 10%
Level of funding: $1,2 million
Project goal: The goal of this study is to construct unique TMA containing prostate cancer samples pre- and post-treatment, or pre and post-recurrence, to provide materials in the validation biomarkers and determination of therapeutic effectiveness
Key personnel receiving salary support from this project: PI 20%, Gomez: 10% effort

Planned:
"Hypoxia and tumor microenvironment to improve cell immunotherapy for prostate cancer". NIH, R01. $250,000/$1,250,000 (direct costs). Period: 07/01/14 – 06/30/19. P.I.

Research Interests

- Immunosenescence: The effects of advanced age on inflammation, immunoregulation, and injury. Age-dependent defects in cytokine networks. Interleukin-6: age-related effects on cellular and systemic immune responses. Hormone replacement and cell mediated immunity in the aged.
- Immunotherapy for prostate cancer: Small molecules as modulators of the tumor microenvironment. Strategies aimed at improving delivery of whole-cell cancer vaccines by improving their antigenicity.
- Cancer biomarker discovery and validation: Hypoxia controlled genes as novel prognostic and theranostic biomarkers in prostate and colorectal cancer.
- Restoring immunity in the aged: Hyperbaric oxygen therapy as mobilizer of stem cells and progenitors in senescent individuals.

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1. Aravena, M., Perez, C., Perez, V., Acuna-Castillo, C., **Gomez, C.R.,** Leiva-Salcedo, E., Nishimura, S., Sabaj, V., Walter, R. and Sierra, F. 2005. T-kininogen can either induce or inhibit proliferation in Balb/c 3T3 fibroblasts, depending on the route of administration. Mech Ageing Dev. 126:399-406
2. **Gomez, C.R.,** Boehmer, E.D. and Kovacs, E.J. 2005. The aging innate immune system. Current Opin Immunol 17:457-462
3. Pérez, V., Velarde, V., Acuña-Castillo, C., **Gomez, C.R.,** Nishimura, S., Sabaj, V., Walter, R., and Sierra, F. 2005. Increased Kinin Levels and Decreased Responsiveness to Kinins During Aging. Gerontol A Biol Sci Med Sci 60:984-990
4. Acuña-Castillo, C., Aravena, C., Leiva-Salcedo, E., Pérez, V., **Gomez, C.R.,** Sabaj, V., Nishimura, S., Pérez, C., Colombo, A., Walter, R. and Sierra, F. 2005. T-kininogen, a cystatin-like molecule, inhibits ERK-dependent lymphocyte proliferation. Mech Ageing Dev 126:1284-91
5. **Gomez, C.R.,** Acuña-Castillo, C., Nishimura, S., Pérez, V., Escobar, A., Sabaj, V., Torres, C., Walter, R. and Sierra, F. 2006. Serum from aged F344 rats conditions the activation of young macrophages. Mech Ageing Dev 127:257-63
6. Pérez, V., Leiva-Salcedo, E., Acuña-Castillo, C., Aravena, M., **Gomez, C.R.,** Sabaj, V., Colombo, A., Nishimura, S., Pérez, C., Walter, R. and Sierra, F. 2006. T-kininogen induces endothelial cell proliferation. Mech Ageing Dev 127:282-89
7. **Gomez, C.R.,** Goral, J., Ramirez, L., Kopf, M. and Kovacs, E.J. Aberrant Acute Phase Response in Aged IL-6 KO mice. 2006. Shock 25:581-85
8. Acuña-Castillo, C., Leiva-Salcedo, E., **Gomez, C.R.,** Pérez, V., Li, M., Torres, C., Walter, R., Murasko, D.M. and Sierra, F. T-kininogen: A biomarker of aging in Fisher 344 rats with possible implications on the immune response. 2006. J Gerontol Biol Sci. 61A:641-49
9. Espinoza, I., **Gomez, C.R.,** Galindo, M. and Galanti, N. Developmental expression pattern of histone H4 gene associated to DNA synthesis in the endoparasitic platyhelminth Mesocestoides corti. 2007. Gene, 386 (1-2): 35-41
10. **Gomez, C.R.,** Birjandi, S., Hirano, S., Cutro, B.T., Baila, H., Nomellini, V. and Kovacs, E.J., Advanced age exacerbates the pulmonary inflammatory response after lipopolysaccharide exposure. 2007. Crit Care Med, 35:246-51
11. Boehmer, E.D., Meehan, M.J., Cutro, B.T., **Gomez, C.R.** and Kovacs, E.J. Aberrant TLR-mediated signal transduction in macrophages from aged mice. (ed., JD Schwarzmeier). 2006. The 6th International Cytokine Conferences. Bologna, Italy, Monduzzi Editore, pp 31-34
12. **Gomez, C.R.,** Nomellini, V., Boehmer, E.D. and Kovacs, E.J. Signal transduction of the aging innate immune system. 2007. Current Immunol Revs, 3: 23-30
13. **Gomez, C.R.,** Plackett, T.P., and Kovacs, E.J., Aging and Estrogen: Modulation of Inflammatory Responses after Injury. 2007. Exp Gerontol, 42:451-56
14. Nomellini, V., **Gomez, C.R.,** and Kovacs E.J. Aging and impairment of innate immunity. 2008. Contrib Microbiol, 15:188-205
15. Karavitis, J., Murdoch, E.L., **Gomez, C.R.,** Ramirez, L., and Kovacs, E.J. Acute ethanol exposure attenuates multiple macrophage functions. 2008. J. Interf. Cytok. Res, 28:413-22
16. Bustos, F., Fernández, C., Duhalde, A., and **Gomez, C.R.,** Motivational profile of the students that enter and remain in the Universidad Diego Portales medical School . A retrospective study. 2008. RECS, 5: 33-40
17. Nomellini, V., Faunce, D.E., **Gomez, C.R.,** and Kovacs, E.J. An age-associated increase in pulmonary inflammation after burn injury is abrogated by CXCR2 inhibition. 2008. J. Leuk. Biol. 83:1493-501

18. **Gomez, C.R.,** Nomellini, V., Faunce, D.E., and Kovacs, E.J. Age and Innate immunity. 2008. Exp Gerontol. 43:718-728. **Note:** This article was one of the top 10 most cited papers in Experimental Gerontology during the period 2008 – 2010. Source www.scopus.com - September 2010
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20. Nomellini, V., **Gomez, C.R.,** Gamelli, R.L., and Kovacs, E.J. Aging, and animal models of systemic insult: Trauma, burn, and sepsis. 2009. Shock. 31:11-20
21. **Gomez, C.R.,** Acuña-Castillo, C., Pérez, C., Leiva-Salcedo, E., Ordenes, E., Oshima, K., Aravena, M., Pérez, V., Nishimura, S., Sabaj, V., Walter, R. and Sierra, F. Defective acute phase response during aging is associated with acute hepatic injury. 2009. J. Gerontol. 63:1299-306
22. **Gomez, C.R.,** Nomellini, V., Baila, H., Oshima K., and Kovacs, E.J. Comparison of the effects of aging and IL-6 on the hepatic inflammatory response in two models of systemic injury: scald injury versus i.p. LPS administration. 2009. Shock. 31:178-84
23. **Gomez, C.R.,** Nomellini, V., and Kovacs, E.J. Part III-10: Immunosenescence and sex hormones, book chapter in: "Handbook on immunosenescence. Basic understanding and clinical applications". 2009. Edited by Tamas Fulop, Katsuiku Hirokawa, Graham Pawelec, and Claudio Franceschi. Springer Netherlands. ISBN: 978-1-4020-9062-2. Pages: 799-831
24. **Gomez, C.R.,** Karavitis, J., Palmer, J.L., Faunce, D.E., Ramirez, L., Nomellini, V., and Kovacs, E.J. Interleukin-6 contributes to age-related alteration of cytokine production by macrophages. 2010. Mediators Inflamm. Article ID:475139
25. Salinas, D.G., Acevedo, C. and **Gomez, C.R.** Modeling a neural network for the learning of the structure-function relationship. 2010. Adv Physiol Educ. 34:158-61.
26. **Gomez, C.R.,** Knutson, G., Bulur, P., Schreiber, C., Vuk-Pavlovic S. Age-dependent response of bone marrow cells to hyperbaric oxygen. 2012. BMC Medicine. 13: 287–297.
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Manuscripts in Preparation

1. **Gomez, C.R.,** Ma T., Knutson, G., Schreiber C.A., Vuk-Pavlovic S., Low oxygen tension as a tool for the development of better prostate cancer cell vaccines
2. **Gomez, C.R.,** Kosari, F., Munz, J.M., Schreiber, C., Knutson, G., Charlesworth, C., Karnes, R. J., Cheville, J., Vasmatzis, G., Vuk-Pavlovic, S. Prognostic value of hypoxia-associated genes in prostate cancer (submitted)
3. Elkhattouti, A., **Gomez, C.R.** Aging Niche in Senescence-Related Pathology (submitted).

Research Reports and Abstracts

1. **Gomez, C.R.,** Walter, R. and Sierra F. 2001. Regulation of gene expression of cytokines during aging. Symposium, "Oxidative stress, cellular responses and modulator factors", XXIV annual meeting of the Chilean Society of Biochemistry and Molecular Biology. Chillán, Chile, p.23
2. **Gomez, C.R.,** Walter, R. and Sierra, F. 2002. Expression of MIP-1α during the acute phase response of aged rats. XVI annual meeting of the Chilean Society of Cellular Biology. Puerto Varas, Chile, p. 22
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Invited Lectures, Seminars, and Presentations

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RESEARCH ARTICLE

Age-dependent response of murine female bone marrow cells to hyperbaric oxygen

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Abstract Consequences of age on the effects of hyperbaric oxygen (HBO) on bone marrow (BM) derived stem cells and progenitors (SCPs) are largely unknown. We treated 2- and 18-month old C57BL/6 female mice by HBO. Hematopoietic stem cells and progenitors, enumerated as colony-forming units in culture, were doubled only in peripheral leukocytes and BM cells of young mice receiving HBO. In old mice colony-forming unit fibroblast numbers, a measure of mesenchymal stromal cells (MSCs) from BM, were high but unaffected by HBO. To further explore this finding, in BM-MSCs we quantified the transcripts of adipocyte early-differentiation genes peroxisome proliferator-activated receptor-γ, CCAAT/enhancer binding protein-β and fatty-acid binding protein 4; these transcripts were not affected by age or HBO. However, osteoblast gene transcripts runt-related transcription factor 2, osterix (OSX) and alkaline phosphatase (AP) were twofold to 20-fold more abundant in MSCs from old control mice relative to those of young control mice. HBO affected expression of osteoblast markers only in old MSCs (OSX gene expression was reduced by twofold and AP expression was increased threefold). Our data demonstrate the impact of aging on the response of BM SCPs to HBO and indicate the potentially different age-related benefit of HBO in wound healing and tissue remodeling.

Keywords Aging · Hyperbaric oxygen · Hematopoietic progenitor cells · Mesenchymal stromal cells

Abbreviations

AP	Alkaline phosphatase
aP2	Fatty-acid binding protein 4
BM	Bone marrow
BMT	Bone marrow transplant
C/EBP-β	CCAAT/enhancer binding protein-β

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CFU-C	Colony-forming units in culture
CFU-F	Colony-forming unit fibroblast
HSCP	Hematopoietic stem cells and progenitors
HBO	Hyperbaric oxygen
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MSC	Mesenchymal stromal cell
OSX	Osterix
PPAR-γ	Peroxisome proliferator-activated receptor-γ
RUNX2	Runt-related transcription factor 2
SCP	Stem cells and progenitor
TNF-α	Tumor necrosis factor α

Background

Allogeneic bone marrow (BM) transplantation (BMT) results in more deaths, more tissue injury and higher pro-inflammatory response in old mice and humans than in the young (Ordemann et al. 2002). Advanced age is accompanied by a marked decrease in the number of $CD34^+$ hematopoietic stem cells and progenitors (HSCPs) and attenuated lymphoid differentiation (Lansdorp et al. 1993; de Haan and Van Zant 1999; Lee et al. 2005). These phenomena suggest that self-renewal and proliferative potential of hematopoietic cells are diminished with age.

Among modalities studied for the potential to mitigate the problems associated with BMT is treatment by hyperbaric oxygen (HBO), a method effective in therapy that requires tissue regeneration (Neuman and Thom 2008). HBO influences tissues by different mechanisms, including modulation of the inflammatory response after BMT (Xiao-Yu et al. 2008) and mobilization of vasculogenic and HSCPs into circulation (Thom et al. 2006; Milovanova et al. 2009).

Although older subjects are more likely to require the benefit of HBO, the role of age on the effectiveness of HBO has not been explored. The need for understanding the role of aging is buttressed by the adverse events caused by the standard methods of HSCPs mobilization by chemotherapeutics and/or growth factors that increase the risk of acute arterial thrombosis, angina, sepsis, and death (Takahashi et al. 1999) in the elderly much more than in the young (Nomellini et al. 2009). The effects of age on the putative HBO-effects on mesenchymal stromal cells (MSCs) and their differentiation potential are unknown. Consequently, we studied the effects of HBO on HSCPs and MSCs in a murine model.

Methods

Animals

Pathogen-free young (2 months) and old (18 months) female C57BL/6 mice from the National Institute of Aging colony at Harlan Laboratories (Indianapolis, IN) were maintained in an environmentally controlled facility at Mayo Clinic for at least one week prior to experiments. Immediately after death, mice were dissected and organs screened for visible tumors and/or gross abnormalities, but none was found. All experimental protocols followed the guidelines in "Principles of Laboratory Animal Care" (NIH publication No. 86-23, revised 1996) and were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Normobaric and HBO treatment

Mice (three to four animals per cage) were placed in an animal hyperbaric chamber (Mechidyne Systems, Inc. Houston, TX). Pressure of pure medical grade oxygen was increased to 2.8 atm absolute (ATA; 283.7 kPa) in the course of 6.5 min. After 90 min of HBO, pressure was reduced to 1.0 ATA (101.3 kPa) in 5.0 min (Quirinia and Viidik 1996; Thom et al. 2006). Control mice were exposed to air or normobaric oxygen to test the evidence that neither pure normobaric oxygen nor hyperbaric air had any effect on HSCPs numbers in circulation (Thom et al. 2006). The animals were exposed to five consecutive daily HBO treatments. Eighteen hours after the last treatment the mice were killed by CO_2 inhalation. The results shown are representative of three independent experiments.

Blood cell count

EDTA-anticoagulated blood was drawn from the right ventricle. Complete blood cell counts were quantified using a Hemavet 850FS cell counter (Drew Scientific, Oxford, CT).

Colony-forming cell assays

Individual hematopoietic colony-forming units in culture (CFU-C) were enumerated in peripheral blood and BM cells. A leukocyte suspension was prepared from EDTA-anticoagulated peripheral blood by lysing erythrocytes with ammonium chloride. BM was obtained by flushing femora with α-minimum essential medium containing 2% fetal bovine serum (FBS). One million leukocytes or 2×10^5 BM cells were plated in 35-mm dishes containing methylcellulose and growth factors (MethoCult GF M3434 assay; StemCell Technologies, Vancouver, Canada) according to manufacturer's protocol and placed at 37°C in humidified air containing 5% CO_2. At day 12, colonies were counted and evaluated using an inverted microscope.

MSC frequency was evaluated by colony-forming unit fibroblast (CFU-F) assay using the complete MesenCult mouse medium 05501 (StemCell Technologies) according to manufacturer's protocol. One million leukocytes or BM cells were plated in duplicate for each mouse and incubated in six-well plates (2.0 mL/well). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. After 10 to 13 days in culture, the medium was decanted, adherent colonies washed with PBS twice, air-dried for 5 min, covered with methanol and incubated at room temperature for 5 more minutes. Then methanol was decanted, colonies were air dried for 5 min and the well covered with Giemsa Staining Solution. After 5 min, the wells were washed with water and air-dried. Colonies were counted using an inverted microscope.

Isolation and culture of splenic macrophages

Splenic macrophages were isolated by plastic adherence (Boehmer et al. 2005; Gomez et al. 2010). Spleens were aseptically removed and the cells disassociated by passing through a nylon mesh in RPMI 1640 medium, supplemented with 5% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and 2 mM glutamine (culture medium; GIBCO-BRL, Grand Island, NY). Following red blood cell lysis with ACK Lysis Buffer (Invitrogen, Carlsbad, CA), white blood cells were counted in a hemocytometer; their viability was determined by trypan blue exclusion. Two million cells/well were seeded in 96-well plates in 200 μL of culture medium. After incubation for two hours at standard tissue culture conditions, non-adherent cells were aspirated and discarded; adherent cells were washed twice with warm phosphate buffer saline. This method resulted in adherent cells that were 98% positive for Mac-3 and Di-I-acetylated low-density lipoprotein uptake (Faunce et al. 1998). Adherent cells were treated in 200 μL of culture medium containing 100 ng/mL LPS from *Escherichia coli 0111:B4* (Sigma, St. Louis, MO). Supernatants were collected after 18 h and stored at −80°C.

Measurement of pro-inflammatory cytokines

Concentrations of tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) in macrophage supernatants were measured by commercial ELISA kits (OptEIA; BD Pharmingen, San Diego, CA) according to manufacturer's instructions. The lower detection limit of the kits was 15.6 pg/mL.

Real-time PCR for early differentiation markers

BM cells were cultured in complete MesenCult mouse medium 05501 for 10 days; the medium was replaced twice a week. Total RNA was isolated using TRIzol Reagent (Invitrogen) and quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). One microgram of RNA was reverse transcribed with Superscript III Reverse Transcriptase (Invitrogen). Following reverse transcription, real-time PCR was performed using LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics, Indianapolis, IN). The forward and reverse primers were designed using the PrimerTime qPCR Assay (Integrated DNA Technologies, Skokie, IL). Primer sequences are shown in Table 1. Each primer master mix consisted of one forward and one reverse primer (10 μM each), SYBR Green I Master Mix (2× concentration), and sterile water. Five μL of the cDNA was aliquoted to each well of the LightCycler 480 Multiwell Plate (Roche Diagnostics); 15 μL of the primer master mix was added to cDNA. Each reaction was run in duplicate in a final volume of 20 μL. In the LightCycler 480 real-time PCR apparatus, the pre-incubation at 95°C took five min and was followed by 45 amplification cycles at 95°C for 30 s and then 60°C for 30 s. The results for individual genes were normalized based on the expression of the housekeeping gene TATA-binding protein (Syed et al. 2010) run in the same plate. Data are expressed as the

Table 1 Primer sequences employed in analysis of transcript levels for early markers of adipocytic and osteoblastic differentiation of mesenchymal stromal cells

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
PPAR-γ	GAGGAGTCCCTTCCCTCATC	TCCTCGAAGGTTAAGGCTGA
C/EBP-β	GTTTCGGGACTTGATGCAAT	GCCCGGCTAGACAGTTACAC
aP2	GAATGTGTTATGAAAGGCGTGAC	AAATTTCCATCCATCCAGGCCTCT
RUNX2	CCAGGAAGACTGCAAGAAGG	TCCTGCATGGACTGTGGTTA
OSX	GGAGGTTTCACTCCATTCCA	TAGAAGGAGCAGGGGACAGA
AP	CACAGATTCCCAAAGCACCT	GGGATGGAGGAGAGAAGGTC
TBP	CTCAGTTACAGGTGGCAGCA	CAGCACAGAGCAAGCAACTC

Markers of adipocytic differentiation: *PPAR-γ* Peroxisome proliferator-activated receptor-γ, *C/EBP-β* CCAAT/enhancer binding protein-β, *aP2* fatty-acid binding protein 4. Markers of osteoblastic differentiation: *RUNX2* runt-related transcription factor 2, *OSX* osterix, *AP* alkaline phosphatase. Housekeeping gene: *TBP* TATA-binding protein

factor ("fold") of change relative to the expression in young control animals.

Statistical analysis

Groups consisted of no more than four mice. The total number of animals studied was 13, 3 and 7 young mice in normobaric air, normobaric oxygen, and HBO, respectively and 9, 3 and 10 old mice in normobaric air, normobaric oxygen, and HBO, respectively. As the number of animals in study groups differed, we analyzed the data using a two-way ANOVA of logarithmically transformed data. p values were obtained as pair-wise comparisons between group means using the Fisher's Least Significant Difference method. Because the overall global p value was 0.009, the Fisher's Protected Least Significant Difference method provided correct control over type I error (false positives); hence, the pair-wise p values less than 0.05 could be considered statistically significant.

Results

HBO affects circulating blood cells in young, but not old mice

To determine the effects of age and HBO on circulating cells, we exposed mice to pure oxygen at 2.8 ATA for 90 min on each of five consecutive days and compared the effects to control mice breathing normobaric air (Table 2). HBO-treated young mice exhibited an increase in lymphocyte and monocyte counts relative to control air-breathing mice ($p < 0.05$), but blood counts of total white blood cells, basophils, eosinophils, red blood cells and platelets did not differ. Peripheral blood counts in old air-breathing mice were similar to young air-breathing mice, except for elevated monocyte and platelet levels ($p < 0.05$). However, HBO did not affect the cell densities in old mice.

Effects of age on hematopoietic progenitors

Because HBO, but not normobaric oxygen, mobilizes SCPs in young mice (Thom et al. 2006; Milovanova et al. 2009), we explored the effects of HBO on SCP mobilization in old mice by determining the number of hematopoietic colony forming units in culture (CFU-C). For additional control, we exposed the animals to normobaric oxygen under conditions otherwise identical to those exposed to HBO. We found no effect of normobaric oxygen compared to normobaric air in the young and old ($p > 0.05$), but the blood of old control mice yielded twice as many CFU-Cs as the blood of young mice ($p < 0.05$; Fig. 1). HBO treatment doubled this number in young mice ($p < 0.05$); the increase in old mice did not reach statistical significance. The number of CFU-Cs from BM cells was higher in old control mice than in young control mice ($p < 0.05$; Fig. 1). HBO effect on BM cells was similar to the effect on circulating cells: it affected young mice ($p < 0.05$), but not old mice. Again, normobaric oxygen had no effect in either group ($p > 0.05$). These results suggest that aging increases the number of CFU-C-generating cells on its own and that HBO has no effect on these cells in old mice.

Table 2 Effects of age on circulating blood cells in young and old mice

	Young		Old	
	Air	HBO	Air	HBO
White blood cells	5.5 ± 0.6	8.5 ± 0.6	6.2 ± 1.1	6.1 ± 0.8
Lymphocytes	4.2 ± 0.5	6.5 ± 0.4*	3.8 ± 0.7	4.0 ± 0.4
Monocytes	0.2 ± 0.0	0.3 ± 0.0*	0.3 ± 0.0**	0.3 ± 0.0
Neutrophils	1.2 ± 0.1	1.7 ± 0.2	1.9 ± 0.4	1.3 ± 0.1
Basophils	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Eosinophils	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Red blood cells	7.8 ± 0.1	8.0 ± 0.4	7.9 ± 0.1	7.3 ± 0.3
Platelets	648 ± 26.8	722 ± 40.9	954 ± 24.1**	900 ± 74.5

Young and old mice were treated by 100% oxygen at 2.8 ATA for 90 min on five consecutive days. Controls breathed normobaric air. Complete blood counts were determined using a Hemavet 850FS cytometer (DREW Scientific, Oxford, CT). All cell numbers are expressed as thousands per μL and shown as mean ± SEM. * Significant compared to animals breathing normobaric air. ** Significant compared to young animals breathing normobaric air. For lymphocytes, $p < 0.05$ for the difference between young controls and young hyperbaric oxygen (HBO)-treated animals; for monocytes, probabilities for comparisons of control and HBO-treated young mice and young control mice versus old control were < 0.05 as was the probability for the difference of platelet numbers between young controls and old controls

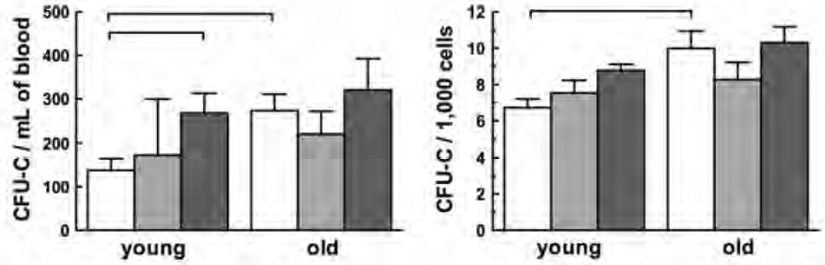
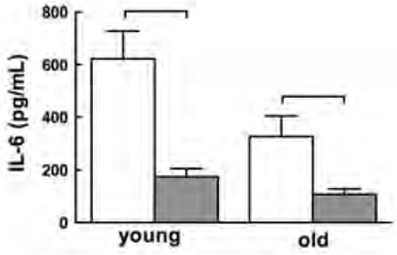


Fig. 1 Hyperbaric oxygen and age affect hematopoietic propensity of circulating and bone marrow cells. Mice breathed normobaric air (controls; *white bars*) or normobaric oxygen (*lightly shaded bars*) or hyperbaric oxygen (HBO; *heavily shaded bars*) for five consecutive days. Eighteen hours after final HBO treatment, animals were killed and hematopoietic stem cells and progenitors (HSCPs) were quantified as colony-forming units in culture (CFU-C) by peripheral blood leukocytes (*left panel*) and bone marrow (BM) cells (*right panel*). Mice were treated in groups of no more than four. The total number of animals studied was 13, 3 and 7 young mice in normobaric air, normobaric oxygen, and HBO, respectively and 9, 3 and 10 old mice in normobaric air, normobaric oxygen, and HBO, respectively. Pooled data for all mice that underwent the same treatment are shown as mean values ± SEM. *Horizontal lines* indicate the differences among groups that are statistically significant ($p < 0.05$)

As normobaric oxygen did not affect proliferation as the ultimate measure of cell function, we focused further studies on the effects of HBO.

HBO suppresses expression of proinflammatory cytokines by activated macrophages

Most CFU-Cs developed from blood and BM cells were CFU-granulocyte/macrophage (data not shown); this indicated that most cells were committed to differentiation into myeloid cells. This observation compelled us to determine the effects of HBO on myeloid cell function. We isolated splenic macrophages, activated them by lipopolysaccharide (LPS) and measured expression of IL-6 and TNF-α, cytokines previously tested in studies of HBO effects (Lahat et al. 1995; van den Blink et al. 2002; Benson et al. 2003; Fildissis et al. 2004; Buras et al. 2006; Thom 2009). We found no detectable cytokines in the media conditioned by LPS-free macrophages (data not shown), but in the presence of LPS macrophages expressed high levels of IL-6 and TNF-α (Fig. 2). In



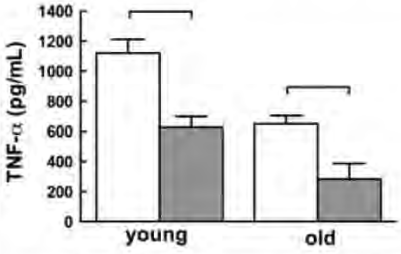


Fig. 2 Hyperbaric oxygen suppresses secretion of inflammatory cytokines. Splenic macrophages obtained from young and old mice shown in Fig. 1 were cultured for 18 h with lipopolysaccharide (LPS; 100 ng/mL). Supernatants were assayed for interleukin-6 (IL-6) (*left panel*) and TNF-α (*right panel*). Data are shown as mean values ± SEM for all mice that underwent the same treatment. *Horizontal lines* indicate the differences among groups that are statistically significant ($p < 0.05$)

agreement with others, macrophages from old mice secreted less of these cytokines (Renshaw et al. 2002; Boehmer et al. 2005; Chelvarajan et al. 2005; Gomez et al. 2010). HBO reduced the mean concentration of IL-6 by 72% in the media conditioned by macrophages from young mice ($p < 0.05$) and by 67% by macrophages from old mice ($p < 0.05$). TNF-α secretion was similarly reduced by 44% ($p < 0.05$) and 56% ($p < 0.05$), respectively. These results show that systemic HBO exposure reduces in vitro pro-inflammatory cytokine expression in macrophages from young and old mice alike.

Effect of age on HBO on MSCs

Based on the recent interest in the potential role of MSCs in regenerative medicine, we set to determine if HBO affects this major group of cells as well. Consequently, we quantified the MSC frequency in peripheral leukocytes and BM by the CFU-F assay. We found no CFU-F-generating cells in the blood of young air-breathing and HBO-treated mice. However, leukocytes of old air-breathing mice yielded low, but detectable numbers of CFU-Fs (1.0 ± 0.8 CFU-F/1×10^6 leukocytes); the number of CFU-Fs did not change by HBO ($p > 0.05$).

In distinction to leukocytes, BM cells gave rise to typical CFU-Fs (Fig. 3). One million BM cells of young mice yielded 4.5 ± 0.8 CFU-Fs (Fig. 4). Surprisingly, HBO doubled the number of CFU-Fs in young mice ($p < 0.05$). Less unexpected was the finding that the number of CFU-F-generating cells in the BM of old mice was fourfold higher relative to young animals ($p < 0.051$), while HBO did not affect

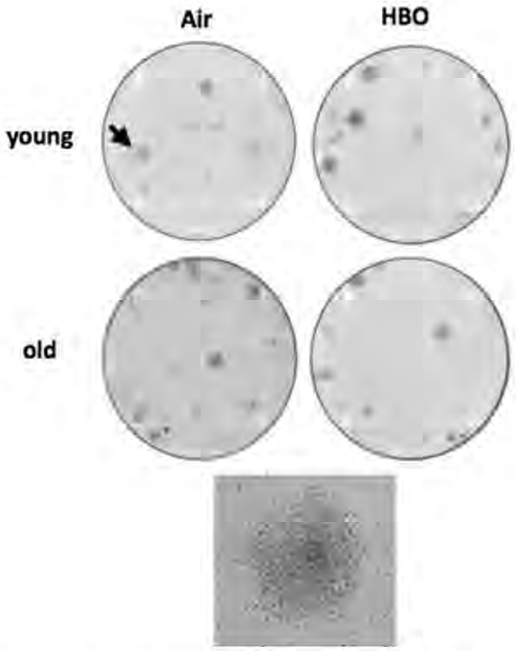


Fig. 3 Age and HBO affect bone marrow-derived MSCs. MSC frequency in leukocytes and BM was evaluated by colony formation. One million leukocytes or BM cells from animals breathing normobaric air or treated by HBO were cultured for up to 13 days and stained by Giemsa when CFU-fibroblast (CFU-F) colonies were counted under the microscope. Shown are representative Giemsa-stained plates of BM-derived CFU-Fs; the arrow in the upper left plate points to the randomly selected colony that is magnified in the square panel to demonstrate the morphology typical for CFU-Fs

that number ($p > 0.05$). These findings suggest that HBO affects BM MSC and that the number of CFU-F-generating cells increases with age.

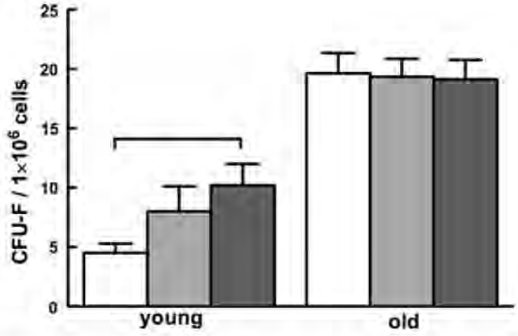


Fig. 4 Hyperbaric oxygen and age affect the frequency of mesenchymal stromal cells in bone marrow. Mesenchymal stromal cells (MSCs) from mice in Fig. 1 were quantified as CFU-fibroblast (CFU-F) colonies. *Horizontal lines* indicate the difference between groups that is statistically significant ($p < 0.05$)

HBO modifies expression of early differentiation genes in MSCs

To gain insight whether age-related and HBO-related changes in CFU-F-generating cells in Figs. 3 and 4 are reflected in the differentiation potential of these cells, we analyzed transcript levels of genes associated with early differentiation into adipocytes and osteoblasts. Genes of adipocytic differentiation (Gregoire et al. 1998) included the peroxisome proliferator-activated receptor-γ (PPAR-γ); CCAAT/enhancer binding protein-β (C/EBP-β); and fatty-acid binding protein 4 (aP2); while those of osteogenic differentiation (van Straalen et al. 1991; Karsenty 2008) were the runt-related transcription factor 2 (RUNX2); osterix (OSX); and alkaline phosphatase (AP). We measured transcript levels by quantitative PCR and for each transcript normalized the data of all groups to its expression level in young air-breathing mice. Interestingly, expression of PPAR-γ and C/EBP-β was affected neither by age nor by HBO, while the level of aP2 transcripts in old mice was elevated relative to young mice ($p < 0.05$); the reduction by HBO did not reach statistical significance (Fig. 5). In contrast, osteoblastic differentiation markers were affected both by age and HBO. The levels of gene transcripts in old mice were threefold, 19-fold and fourfold higher for RUNX2, OSX and AP, respectively ($p < 0.05$ for all three transcripts).

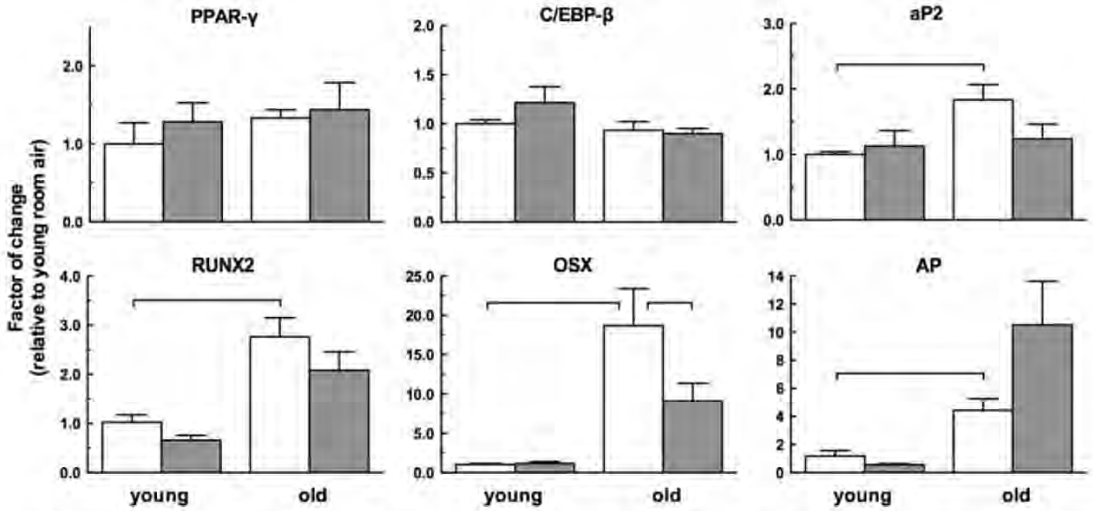


Fig. 5 Hyperbaric oxygen modulates transcription of osteogenic, but not adipogenic differentiation genes in bone marrow mesenchymal stromal cells of old mice. Transcripts associated with early adipogenic differentiation [peroxisome proliferator-activated receptor-γ (PPAR-γ); CCAAT/enhancer binding protein (C/EBP)-β; and, fatty acid binding protein 4 (aP2)]; and early osteogenic differentiation [runt-related transcription factor 2 (RUNX2); osterix (OSX); and, alkaline phosphatase (AP)] were quantified by real-time PCR in bone marrow mesenchymal stromal cells obtained from mice in Fig. 1. *Horizontal lines* indicate the differences among groups that are statistically significant ($p < 0.05$)

While the effects of HBO on transcript levels were marginal in young mice, they were prominent in the old. There was no change in RUNX2 levels, levels of transcripts for OSX were reduced by one half ($p < 0.05$) in contrast to doubling of AP transcripts (not statistically significant). Overall, these results indicate age and HBO affect the expression of osteoblastic early differentiation genes in MSCs.

Discussion

The purpose of this work is to probe the effects of senescence on the ability of HSCPs and MSCs to respond to HBO. The impetus for the study is the need to understand if efficacy of the standard HBO therapy protocols depends on age and, hence, the protocols might have to be tailored to patient age. Based on the general understanding of differences in HSCPs and MSCs between the young and the old (Lansdorp et al. 1993; de Haan and Van Zant 1999; Lee et al. 2005; Sethe et al. 2006; Gazit et al. 2008; Roobrouck et al. 2008), the underlying hypothesis has been that HBO may affect HSCPs and MSCs in an age-dependent manner.

For this study we selected 2-month old and 18-month old mice. Based on the allometric relationships between body size and expected longevity, the younger group would be roughly equivalent to human adolescents, while the older to an octogenarian (Lindstedt and Calder 1981). In this study we focused on a single gender of mice (females); additional experiments in males should define the effect of age and gender on HBO-mediated mobilization of SCPs. Some gender-specific differences might be anticipated based on gender-related response of old mice to stressors (Gomez et al. 2009).

Using the selected model of senescence, we confirmed that the levels of CFU-C-forming cells in the blood and marrow of old mice were higher than in the young mice. A pertinent finding is that HBO affected the proliferative capacity of HSCPs and MSCs in the young, but not in old mice, in line with the observation that systemic hyperoxia enhances proliferation of human (Thom et al. 2006) and murine BM-derived HSCPs (Thom et al. 2006; Milovanova et al. 2009). After a single HBO treatment Thom et al. detected a twofold increase in CFU-Cs in murine peripheral blood leukocytes and BM cells. They found HSCP mobilization by measuring expression of CD34 and Sca-1 in peripheral blood cells (Thom et al. 2006; Milovanova et al. 2009); our results confirm the latter finding by colony forming and demonstrate that the cells are functional and add considerable weight to evidence that HBO mobilizes HSCPs.

Mobilizing agents induce rapid emigration of stem cells from the BM. However, under most circumstances mobilization requires cell proliferation in the BM (Nakamura et al. 2004). The higher basal level of HSCPs in old mice could be associated with the age-related expansion of the SCP pool that originates in the increased autonomous cell renewal capacity (Gazit et al. 2008). In contrast, old age is linked to a decline in SCP function (Sudo et al. 2000; Geiger and Van Zant 2002), including less efficient hematopoiesis (Chen et al. 2000), reduced differentiation potential (Rossi et al. 2005) and impaired homing (Xing et al. 2006). Altogether, these studies suggest that age-related reduction of HSCP activity could be offset by the increase in HSCP numbers (Gazit et al. 2008). Our results are in line with other phenomena in aging, e.g., the pro-inflammatory phenotype, even in the absence of insult ("inflamm-aging") that results in an inherent activation rendering the system less able to respond to perturbation (de Haan and Van Zant 1999; Franceschi et al. 2000; Gomez et al. 2005).

Our finding that HBO does not mobilize HSCPs in the old is at variance with mobilization by granulocyte-colony stimulating factor (Xing et al. 2006) that reduces HSCP adhesion to BM stroma even in the old (Geiger and Van Zant 2009). This difference suggests qualitatively and/or quantitatively distinct mechanisms in HBO-mediated and G-CSF-mediated HSCP mobilization in old mice. In response to HBO, nitric oxide levels increase and trigger a cascade mechanism that mobilizes SPCs from BM by the release into circulation of cytokines such as cKit ligand (stem cell factor, SCF; Thom et al. 2006). The effects of aging on this mechanism are unknown, but HSPC mobilization by chemotherapeutics and/or growth factors increases the risk of acute arterial thrombosis, angina, sepsis, and death (Takahashi et al. 1999) in the old much more than in the young (Nomellini et al. 2009). Consequently, it will be important to clarify the effects of aging on the components of the proposed HBO-mediated cascade involved in SCP mobilization. Additional insight might be gained from studies of the effects of aging and HBO on the expression of

SDF-1 and CXCR4, the critical regulators of SPC function and homing, and of nitric oxide-mediated mechanism of HSCP mobilization by HBO.

Aging increases the differentiation potential of myeloid cells [reviewed in (Linton and Dorshkind 2004)], but results also in their functional defects [reviewed in (Gomez et al. 2008)]. Our studies confirmed that LPS-activated macrophages from old mice express less proinflammatory cytokines IL-6 and TNF-α relative to young cells (Renshaw et al. 2002; Boehmer et al. 2005; Chelvarajan et al. 2005; Gomez et al. 2010). Interestingly, HBO reduced IL-6 and TNF-α expression to a similar extent in the young and the old, in line with some (Benson et al. 2003; Buras et al. 2006; Thom 2009), but not all observations (Lahat et al. 1995; van den Blink et al. 2002; Fildissis et al. 2004). Apparently, HBO can control acute inflammation following injury and sepsis (Huang et al. 2005; Oter et al. 2005; Neuman and Thom 2008), but its effects on increased morbidity and mortality associated with aberrant inflammatory responses in the old are largely unknown and, therefore, very much worthy of additional research. While HBO-mediated mobilization of HSCPs into circulation has been known for some time (Thom et al. 2006), HBO effects on MSCs have not been studied. We found no CFU-F-forming cells among peripheral blood leukocytes under any condition, except in old mice where we did detect low levels of these cells. The significance of this observation will require further study. However, interestingly in BM, HBO increased the CFU-F number in young mice.

MSCs and the effects of aging on them (Sethe et al. 2006; Roobrouck et al. 2008) are gaining interest because of the putative applicability of MSCs in regenerative therapy and tissue engineering (Parekkadan and Milwid 2010). We found detectable levels of circulating MSCs (measured by the ability to generate CFU-F colonies) only in old air-breathing mice. In the BM, these cells were detectable in young mice and at a fourfold higher level in old mice. This result differs from the reports of age-related decrease in the total number of CFUs or the absence of age effects [reviewed in (Sethe et al. 2006)]. Further MSC evaluation by techniques like flow cytometry may be needed to detect fractions of MSCs especially sensitive to the culture conditions used in CFU-F assays and to settle the discord among the results.

The effects of oxygen tension on MSCs have not been clearly established either. On one side, hypoxia decreased proliferation and differentiation of BM-derived MSCs (Mohyeldin et al. 2010). On the other side, HBO-stimulated bone formation and healing (processes involving MSCs) did not enhance the osteogenic ability of MSCs in spinal fusion in a rabbit model (Fu et al. 2010). We found that HBO increased the number of CFU-Fs in the young BM. We demonstrated that young MSCs retained their differentiation potential (measuring adipogenic differentiation; data not shown), but that HBO had no effect on this process. This observation adds to those who suggest that in young mice HBO can change the numbers of BM-derived MSCs without affecting their differentiation potential (Fu et al. 2010).

Lineage commitment of MSCs is controlled by an array of intracellular and extracellular signals in the BM milieu, including the activation of phenotype-specific transcription factors (Karsenty 2008). Our results show that transcript levels of genes involved in adipogenic differentiation were unaffected by age, but that transcript levels of early osteoblastic differentiation genes were higher in old MSCs. These results differ from description of higher levels of aP2 transcripts (an adipocyte differentiation gene) in MSCs from old mice (no gender specified) and lower levels of transcripts of osteoclast-specific transcription factors including RUNX2 (Moerman et al. 2004). However, our findings are in line with the expression of significantly higher transcript levels for all osteoblast differentiation marker genes in hematopoietic lineage-negative (lin^-) cells from old female C57/BL6 mice (Syed et al. 2010). Since lin^- cells are highly enriched in osteoblastic progenitors that can mineralize in vitro, form bone in vivo, and express bone-related genes, our results are comparable with the demonstration of aging effects on osteoblast progenitors (Syed et al. 2010). Quantifying the baseline expression of osteogenic and adipogenic differentiation markers provided a snapshot of the effects of aging and HBO on the MSC differentiation potential. Expression of these genes during MSC differentiation coupled with other differentiation assays (e.g., in vitro mineralization and in vivo bone formation for osteoblasts and in vitro lipid formation for adipocytes) will provide more definite evidence for the effects of age and HBO on lineage commitment by MSCs.

Observation of MSC-restricted expression of early differentiation genes affected by HBO in old mice only warrants in-depth studies of the involved intracellular signaling networks. Study of signaling involving extracellular mediators, in particular the transforming growth factor-β, bone morphogenetic proteins 2 and 4 with a known role in controlling adipocyte versus osteoblast formation (Gregoire et al. 1998; Karsenty 2008) will help define the mechanisms involved in the effects of HBO in MSCs, particularly in the old.

In a murine model of aging we studied the effects of HBO on HSCPs and MSCs. We demonstrated that aging affected the ability of mice to mobilize HSCPs from the BM and suggest an age-related defect in mobilization by HBO; mechanistic aspects of this phenomenon require further elucidation. Our results suggest that HBO therapy protocols may have to be adjusted by age or eventually individualized. In addition, these results indicate the potentially different benefit of HBO in wound healing and tissue remodeling in the old and the young.

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Original article

Dysregulation of Neutrophil CXCR2 and Pulmonary Endothelial ICAM-1 Promotes Age-Related Pulmonary Inflammation

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ABSTRACT: We have previously demonstrated that aging is associated with prolonged pulmonary inflammation in a murine model of thermal injury. To further investigate these observations, we examined lung congestion, markers of neutrophil chemotaxis and adhesion, and lung endothelia responses in young and aged mice following burn injury. Analysis of lung tissue and bronchoalveolar lavage fluid 24 hours after injury revealed that more neutrophils accumulated in the lungs of aged mice ($p<0.05$), but did not migrate into the alveoli. We then sought to determine if accumulation of neutrophils in the lungs of aged mice was due to differences in the peripheral neutrophil pool or local changes within the lung. Following burn injury, aged mice developed a pronounced peripheral blood neutrophilia ($p<0.05$) in comparison to their younger counterparts. In aged animals, there was a reduced frequency and mean fluorescent intensity of neutrophil CXCR2 expression ($p<0.05$). Interestingly, in uninjured aged mice, peripheral blood neutrophils demonstrated elevated chemokinesis, or hyperchemokinesis, ($p<0.05$), but showed a minimal chemotatic response to KC. To determine if age impacts neutrophil adhesion molecules, we assessed CD62L and CD11b expression on peripheral blood neutrophils. No age-dependent difference in the frequency or mean fluorescent intensity of CD62L or CD11b was observed post-burn trauma. Examination of pulmonary vasculature adhesion molecules which interact with neutrophil selectins and integrins revealed that intracellular adhesion molecule-1 (ICAM-1) was elevated in aged mice at 24 hours after burn as compared to young mice ($p<0.05$). Overall, our data suggests that age-associated pulmonary congestion observed following burn injury may be due to differences in lung endothelial adhesion responses that are compounded by elevated numbers of hyperchemokinetic circulating neutrophils in aged mice.

Key words: Adhesion; Aging; Chemotaxis; Neutrophils; Lung; Burn injury

While the mortality of burn patients over the age of 65 has improved over the last few decades, clinical outcomes are still very poor [1, 2]. As the proportion of elderly individuals continues to rise, this issue will translate into a greater socioeconomic burden. It is therefore important that new treatment strategies are developed to minimize the effects of age on the response to burn and other forms of traumatic injury.

Similar to other insults which lead to systemic inflammation, the development of pulmonary complications, such as pneumonia and acute respiratory distress syndrome, are often the most serious threat to

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the burn patient and are especially detrimental to the elderly [3-5]. In contrast to most other organs in the body, the lung has two potential routes for an inflammatory insult: through the airway and through the bloodstream. Interestingly, the pathogenesis of pulmonary inflammation is dependent on where the stimulus is located. When an inflammatory source is located in the airway, a rapid neutrophil recruitment to the alveolar space is observed [6-12]. However, when the source originates elsewhere in the body and disseminates through the blood, neutrophil accumulation within the pulmonary vasculature occurs, but cells do not migrate into the alveoli; here, the neutrophils are said to be "sequestered" in alveolar capillaries [13-15] The difference between these two host responses are established by multiple factors including chemokine gradients and expression of adhesion molecules [7, 16, 17].

Regardless of the mechanism of pulmonary inflammation, neutrophil chemokines are important in neutrophil recruitment in response to local or systemic injury. In mice, the main chemokines involved in the process are macrophage inflammatory protein-2 (MIP-2, or CXCL2) and KC (or CXCL1), analogues of human growth-related oncogene α and β, respectively [18, 19]. These chemokines both bind to the receptor, CXCR2, on neutrophils [20, 21]. Not only do these chemokines act to stimulate neutrophil chemotaxis towards an inflammatory stimulus, but also to induce firm adhesion to the endothelium and to mediate diapedesis [19, 21, 22]. These studies also imply that there is a sequential order of signaling required for neutrophil diapedesis involving both selectins (CD62L) and integrins (CD11/CD18) [19, 23]. Following ligation of the neutrophil chemotatic receptor CXCR2, activation of the small GTPase: Ras-related protein-1 (Rap1) and phospholipase C (PLC) promotes upregulation of neutrophil selectins and integrins [24-26]. Subsequent interaction and cross-linking of CD62L, further upregulates integrin activity and allows neutrophils to roll and loosely adhere to the vascular endothelium [27, 28]. Together, this results in clustering of CD11/CD18, as well as other adhesion molecules, and promotes firm adhesion of the neutrophils to the endothelium via intracellular adhesion molecule-1 (ICAM-1) [17]. Neutrophils can then interact with chemokines immobilized on the apical side of endothelial cells and begin the process of diapedesis [21, 29, 30].

Previously, our laboratory was the first to show that aged mice have prolonged pulmonary inflammation after burn injury compared to young mice with a comparable insult, marked by neutrophil accumulation and increased KC levels [31]. Herein we demonstrate that following burn trauma, aged mice have an elevated number of neutrophils in both the peripheral blood and lung that is unrelated to differences in neutrophil chemotatic or adhesion markers. Moreover, our data suggest that an age-associated elevation in lung endothelial ICAM-1 may promote the observed pulmonary congestion.

MATERIALS AND METHODS

Animals

Young (2-6 months) and aged (18-22 months) female BALB/c mice were obtained from the National Institute of Aging colony at Harlan Laboratories (Indianapolis, IN) and maintained on a 12 hour light/dark cycle with standard laboratory rodent chow and water ad libitum. All experimental procedures were performed according to the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals, National Institutes of Health, and approved by the Animal Care and Use Committee at Loyola University Medical Center.

Induction of Burn Injury

As previously described, mice were anesthetized with pentobarbital (50 mg/kg i.p.), shaved and placed into a plastic template designed to give a 15% total body surface area, full-thickness dorsal scald injury when immersed in a boiling water bath for 8 seconds [31, 72, 73]. As a control, separate groups of young and aged mice received a sham injury, in which they were administered anesthesia and shaved, but immersed in a room temperature water bath. Immediately following injury, the mice received warm saline resuscitation and their cages were placed on heating pads until fully recovered from anesthesia. Mice were sacrificed using CO_2 inhalation and cervical dislocation. In addition, all mice—including those which died before the time of sacrifice—were examined for visible tumors and, if found, were removed from the study.

Bronchoalveolar Lavage

To determine the cell populations in the alveolar space, bronchoalveolar lavage (BAL) was performed on young and aged mice 24 hours after receiving either a burn or a sham injury [6][2]. Immediately following sacrifice, the trachea was exposed and a small incision was made just below the cricothyroid cartilage. The trachea was then cannulated using 22 gauge needles and 1 ml of phosphate buffered saline was repeatedly injected until 5 ml of fluid was recovered for each animal. BAL cells were immunostained for flow cytometry analysis, as described below.

Isolation of Peripheral Blood Neutrophils

Blood was taken via cardiac puncture of the left ventricle. For chemotaxis assays, samples were diluted

1:1 in Hank's Buffered Saline Solution (HBSS), layered on Histopaque 1083 (Sigma, St. Louis, MO), and centrifuged at 400 g for 30 minutes at 20°C without the brake applied. The monocyte and plasma layers were aspirated, leaving granulocytes and erythrocytes. Samples were resuspended in HBSS and 3% dextran was added to sediment the erythrocytes. After 45 minutes at room temperature, the top layer containing granulocytes was removed and centrifuged at 300 g for 5 minutes. Any remaining erythrocytes were lysed using ACK buffer (Invitrogen, Carlsbad, CA).

Chemotaxis Assay

Neutrophils were isolated as described above. Chemotaxis assays were then performed as described by others [52, 74]. Neutrophils were centrifuged and resuspended in 40 μM of Cell Tracker Green (Invitrogen) in chemotaxis media containing HBSS, 25 mM HEPES and 1% BSA at 10^6 cells/ml. The cells were then incubated in the dark for 45 minutes at 37°C and 5% CO_2. After washing, the cells were resuspended in chemotaxis media at 10^6 cells/ml. The bottom wells of a chemotaxis chamber (NeuroProbe, Gaithersburg, MD) were filled with various concentrations of recombinant mouse KC (R&D Systems). A separate set of wells were filled with media alone as a negative control or 10^{-7} M fMLP (Sigma) as a positive control. Another set of wells were filled with sample inputs to determine the fluorescence of the starting cell suspension. A filter membrane containing 8 μm pores (NeuroProbe) was then placed over the wells and cell suspensions were added to the upper side of the membrane at 10^6/ml. Samples were incubated for 60 minutes at 37°C and 5% CO_2. Cell suspensions were then aspirated off the top membrane and 20 μM EDTA was added to the upper side of the membrane for 15 minutes to allow any cells adhering to the membrane to detach. The membrane was then removed and the fluorescence of the bottom wells was measured in a fluorescence spectrophotometer. The percent of cells migrating was determined by comparing the fluorescence of the cells in the sample wells to that of the input wells.

Flow Cytometry

Analyses utilizing flow cytometry were performed as previously described [75, 76]. Cells were washed with HBSS and blocked with anti-CD16/32 antibody for 30 minutes at 4°C. Cells were then stained for 30 min at 4°C using anti-mouse antibodies at saturating concentrations, washed twice and fixed with 1% paraformaldehyde. Fluorescence was measured by flow cytometry (FACSCanto, BD Biosciences, San Jose, CA). Anti-mouse antibodies were used at the following concentrations: 2 μg/ml of PE-conjugated rat anti-mouse Gr-1 (Invitrogen), 20 μg/ml of APC-conjugated rat anti-mouse F4/80 (eBioscience, San Diego, CA) 10 μg/ml of FITC-conjugated rat-anti mouse Gr-1 (eBioscience), 12.5 μg/ml of PE-conjugated rat anti-mouse CXCR2 (R&D Systems, Minneapolis, MN), and 10 μg/ml of PE-conjugated rat anti-mouse CD11b (eBioscience). Flow cytometry data were analyzed using FlowJo (Tree Star, Inc., Ashland, OR).

Immunofluorescence

The lungs were removed at the time of sacrifice, inflated with 25% O.C.T. freezing medium, and embedded for frozen sectioning as previously described [31]. Tissue sections were fixed in acetone and blocked with normal goat serum. To determine neutrophil content in lungs, sections were first incubated with 1 μg/mL of rat anti-Gr-1 antibody (Invitrogen, Carlsbad, CA) followed by 4 μg/ml of goat anti-rat IgG conjugated to Alexa Fluor 488 (Invitrogen). Since Gr-1 can also be found on certain macrophage populations [3, 4], the sections were dual-stained with 0.2 μg/mL of biotinylated anti-MOMA-2 antibody (BMA Biomedicals, Augst, Switzerland), a pan-macrophage marker, and detected with 2 μg/ml of Cy3 Streptavidin (Invitrogen). Using fluorescent microscopy, the total number of neutrophils (designated as Gr-1^+ MOMA-2^- cells) were counted across 10 high power fields for each animal [5]. Data are expressed as mean number of neutrophils counted in ten 400x fields ± SEM. The total tissue area across which cells were counted was quantified and determined to be consistent between animals in all treatment groups (data not shown).

To determine the expression of endothelial ICAM-1 in the lungs after burn or sham injury, sections were incubated with 0.25 μg/ml of Armenian hamster anti-mouse ICAM-1 (BD Pharmingen, San Diego, CA), followed by 3 μg/ml of goat anti-Armenian hamster IgG conjugated to Cy3 (Jackson Immunoresearch, West Grove, PA). Since ICAM-1 is also expressed on lung epithelium, sections were dual stained with 0.16 μg/ml of rat anti-mouse platelet-endothelial cell adhesion molecule-1 (PECAM-1, BD Biosciences), and subsequently by 4 μg/ml of goat anti-rat IgG conjugated to Alexa Fluor 488 (Invitrogen). Expression of ICAM-1 on lung endothelium was determined by quantifying the total area of ICAM-1 and PECAM-1 colocalization across ten 400x fields per animal. Colocalization is expressed as the area of ICAM-1^+PECAM-1^+ (μm^2) staining. All fluorescent images were acquired using a Zeiss Axiovert 200 microscope (Zeiss, Germany).

Table 1. Neutrophil localization in lungs at 24 hours after burn

	Young		Aged		
Method	**Sham**	**Burn**	**Sham**	**Burn**	
Total # Gr-1$^+$ MOMA-2$^+$[a] cells in 10 fields by immunofluorescence	16.0 ± 2.4	16.1 ± 2.4	12.8 ± 2.5	63.3 ± 11.5	*
% Gr-1$^+$ F4/80$^+$ cells[b] in lung homogenates by flow cytometry	2.0 ± 0.4	1.8 ± 0.3	2.1 ± 0.2	11.8 ± 3.0	‡
% Gr-1$^+$ F4/80$^+$ cells[c] in BAL by flow cytometry	4.1 ± 3.5	0.4 ± 0.1	7.0 ± 2.7	0.5 ± 0.2	§

[a] *Total numbers of Gr-1$^+$ MOMA-2$^+$ cells in lungs of young and aged animals at 24 hours after sham or burn injury were counted in sections of lung tissue. Data are shown as the average number of cells counted in ten 400x fields for each group ± SEM. N = 8-14 mice per group; *p<0.05 compared to all other groups.*

[b] *All five lung lobes were homogenized in HBSS as described above. Cells were stained for Gr-1 and F4/80 as described and analyzed by flow cytometry. Data are shown as the average percent of Gr-1$^+$ F4/80$^+$ cells in the whole cell suspension ± SEM. N = 6-8 mice per group; ‡p<0.05 compared to all other groups.*

[c] *Lungs were lavaged with 1 ml of saline until 5 ml of sample was collected. Cells were spun down and stained with Gr-1 and F4/80 as described and analyzed by flow cytometry. Data are shown as the average percent of Gr-1$^+$ F4/80$^+$ cells in BAL ± SEM. §p<0.05 compared to age-matched control.*

Statistical Analysis

Data were analyzed using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA) and are expressed as mean ± SEM. A one-way ANOVA with Tukey's post-hoc was used to determine statistical differences between all groups. For comparisons of two groups, an unpaired Student's t-test was used. Statistical significance was set at $p<0.05$.

RESULTS

Localization of neutrophils in the lungs of aged mice after burn

We previously reported that neutrophils are cleared from the lungs of young mice at 24 hours after burn while these cells are persistently elevated in the lungs of aged mice receiving the same injury [31]. Multiple methods were employed to determine the pulmonary compartment in which the neutrophils are located in the lungs of young versus aged mice at 24 hours after burn. Lung sections were co-immunostained with anti-Gr-1 and anti-MOMA-2 antibodies, and Gr-1$^+$MOMA-2$^+$ cells were considered neutrophils [32, 33]. Quantification of the number of neutrophils in lung sections from each group is shown in Table 1. At 24 hours after burn injury, there was a 4-fold increase in neutrophil numbers in aged mice subjected to burn injury compared to sham and young burn groups ($p<0.05$). These data further corroborate our previous study in which neutrophils in aged mice were sequestered in the lung interstitium or vasculature at 24 hours after burn injury [31]. Consistent with our previously published studies [31], lung neutrophil numbers did not differ between young sham and young burn mice 24 hours post injury (Table 1). These observations were confirmed by flow cytometry of whole lung cell suspensions. However, instead of using anti-MOMA-2 antibody for alveolar macrophages, anti-F4/80 antibody was used, since it detects circulating monocytes as well [33-35]. As shown in Table 1, while neutrophils (Gr-1^{hi} F4/80$^-$ cells) in the lungs of young, burn-injured mice were similar to sham-injured animals, those from aged, burn-injured mice were 6 times greater than sham controls and young burn animals ($p<0.05$).

We then sought to determine if neutrophils from either young or aged mice transmigrated and persisted in the alveolar space following burn trauma. Lungs were lavaged and BAL fluid was analyzed by flow cytometry (Table 1). As expected, BAL cells of both young and aged sham-injured mice were predominantly macrophages (F4/80$^+$ Gr-1$^-$ cells) and did not differ between treatment groups (data not shown). Neutrophils (Gr-1^{hi} F4/80$^-$ cells) comprised approximately 4% in the young and 7% in the aged of cells recovered from BAL as determined by flow cytometry. Interestingly, the proportion of neutrophils in the BAL decreased in both young and aged mice after burn, although this was only statistically significant in the aged burn-injured mice compared to age-matched controls ($p<0.05$). Together, these data indicate that the neutrophils are sequestered in the lung interstitial and/or vascular space of aged mice 24 hours after injury, but are absent in the alveolar space.

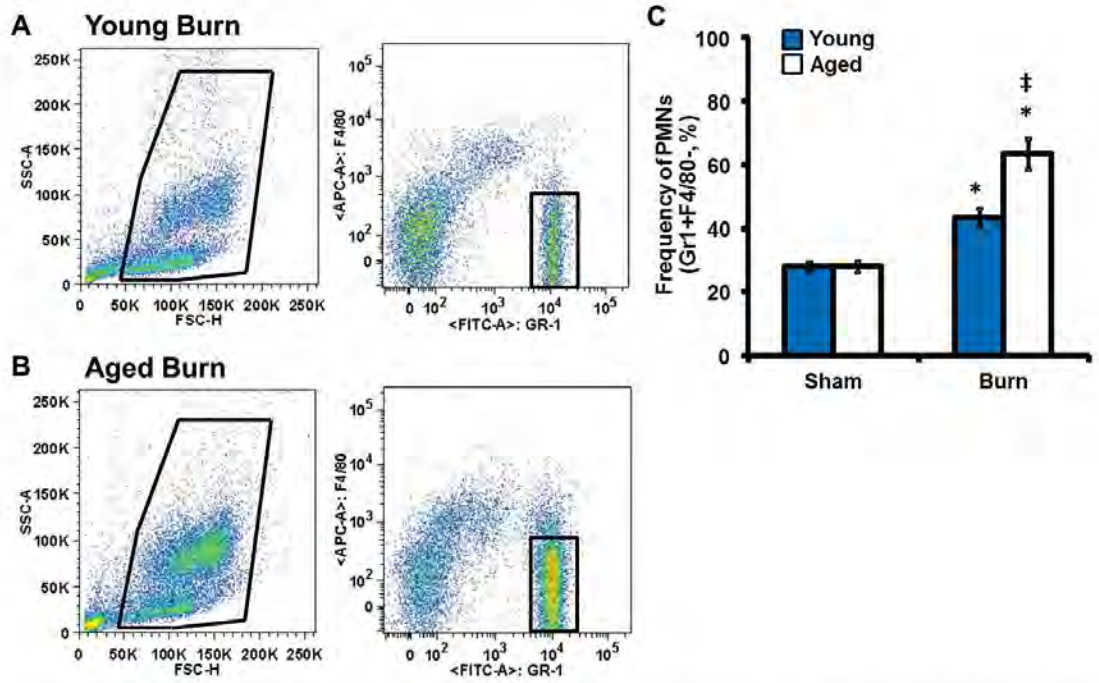

Figure 1. Peripheral blood neutrophil profile. Peripheral blood cells were stained with anti-Gr-1 and anti F4/80 antibodies and analyzed by flow cytometry. Representative live and neutrophil ($Gr\text{-}1^{+}F4/80^{-}$) for young (A) and aged (B) mice. (C) Total neutrophil frequency in whole blood. Data are shown as mean ± SEM. N = 8-15 mice per group; *$p<0.05$ compared to sham controls; ‡$p<0.05$ compared to young burn by one-way ANOVA.

Circulating neutrophil numbers and CXCR2 expression

Considering the heightened accumulation of neutrophils in the lungs of aged mice, we sought to examine the peripheral blood neutrophil population as circulating numbers of neutrophils or altered expression of the chemotatic receptor, CXCR2, on neutrophils may contribute to these observations (Figure 1). Following burn injury, both young and aged mice exhibit elevated percentages of circulating neutrophils in relation to sham controls (Figure 1A-C, $p<0.05$). However, aged mice demonstrate a ~20% increase in peripheral blood neutrophils as compared to young burn mice (Figure 1A-C, $p<0.05$).

As CXCR2 mediates the neutrophil chemotatic response to KC, previously shown to be significantly elevated in lungs of aged mice 24 hours post injury [31], we sought to determine the frequency and mean fluorescent intensity (MFI) of CXCR2 expression in peripheral blood neutrophils (Figure 2). Interestingly, following burn injury there is a reduction in both the percentage of neutrophils expressing CXCR2 and the magnitude of CXCR2 expression in aged mice (Figure 2A-C, $p<0.05$). These data suggest that aging may alter the CXCR2 axis in the setting of trauma. Moreover, the prolonged elevation of KC in the lungs of aged mice at 24 hours post burn [31] may contribute to the observed downregulation of CXCR2 and alter neutrophil migration.

CXCR2-mediated neutrophil chemotaxis

Others have shown that pretreatment of neutrophils with various inflammatory stimuli can inhibit migration through activated endothelium *in vitro* as a result of

CXCR2 desensitization [6]. Coinciding with these studies, a basal pro-inflammatory state has been observed in the setting of advanced age and these mediators can remain elevated in aged mice in trauma models [14, 31, 36, 37]. Given the results of the previous study, we hypothesized that a migratory defect in neutrophils from aged mice may play a role in neutrophil accumulation in the lungs of aged mice after acute burn injury. Chemotaxis of isolated peripheral blood neutrophils from uninjured young and aged mice was observed in a transwell system in response to KC. This particular chemokine was chosen because our previous data indicated that there was an age-related increase between levels of KC lung homogenates and numbers of neutrophils in lung sections after burn injury [31][1]. Since pulmonary levels of MIP-2 in previous studies did not show age differences, this chemokine was not assessed. In the absence of any stimulant, migration of neutrophils from aged mice was significantly higher compared to young mice (Figure 3, $p<0.05$). In the presence of physiologic doses of KC, neutrophils from young mice had a robust response compared to unstimulated controls ($p<0.05$). However, no chemotatic response was generated in neutrophils from aged mice in response to the same increasing doses of KC. Figure 3B shows the same data re-expressed as percent control to obviate the differences in trends between the two age groups. These data suggest that neutrophils from uninjured aged mice exhibit hyperchemokinesis, but impaired directional migration in response to a stimulus. The lack of stimulus-mediated motility may be due to defects in migratory mediators downstream of CXCR2 [17, 30, 38, 38].

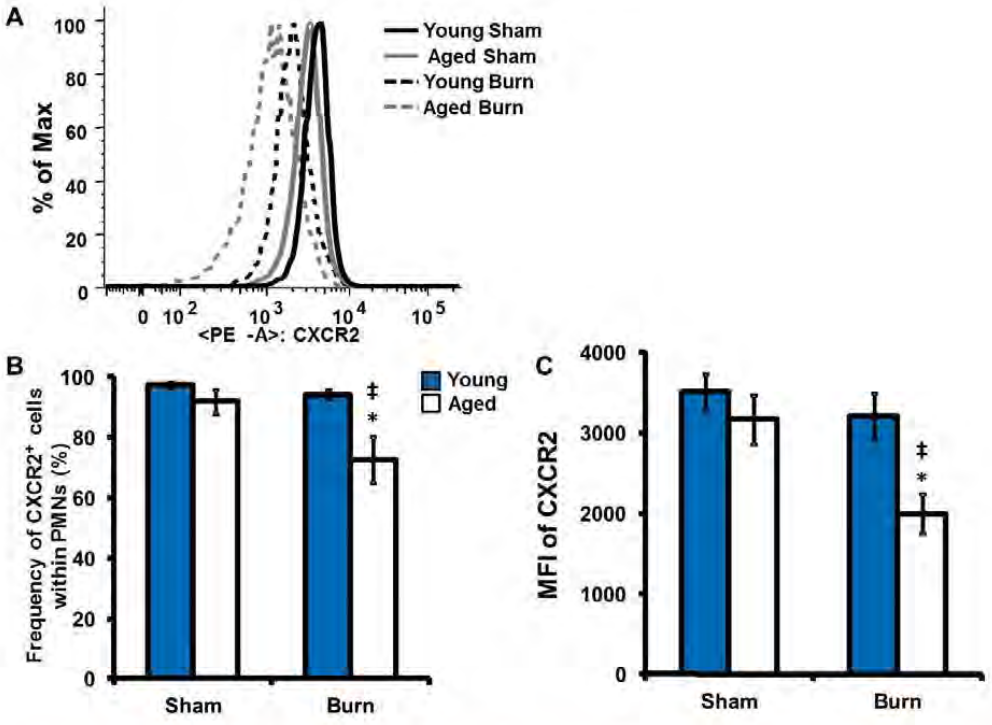

Figure 2. Peripheral blood neutrophil CXCR2 expression. Peripheral blood cells were stained with anti-Gr-1, anti F4/80 antibodies and anti-CXCR2 antibodies and analyzed by flow cytometry. (A) Representative histogram of CXCR2 neutrophil population with young sham- thick black, aged sham- thick gray, young burn- dotted black and aged burn- dotted gray. (B) Frequency of $CXCR2^+$ cells within the neutrophil population; young- dark gray bars and aged- white bars. (C) Mean fluorescent intensity of CXCR2 within $CXCR2^+$ neutrophils. Data are shown as mean ± SEM. N = 8-15 mice per group; *$p<0.05$ compared to sham controls; ‡$p<0.05$ compared to young burn by one-way ANOVA.

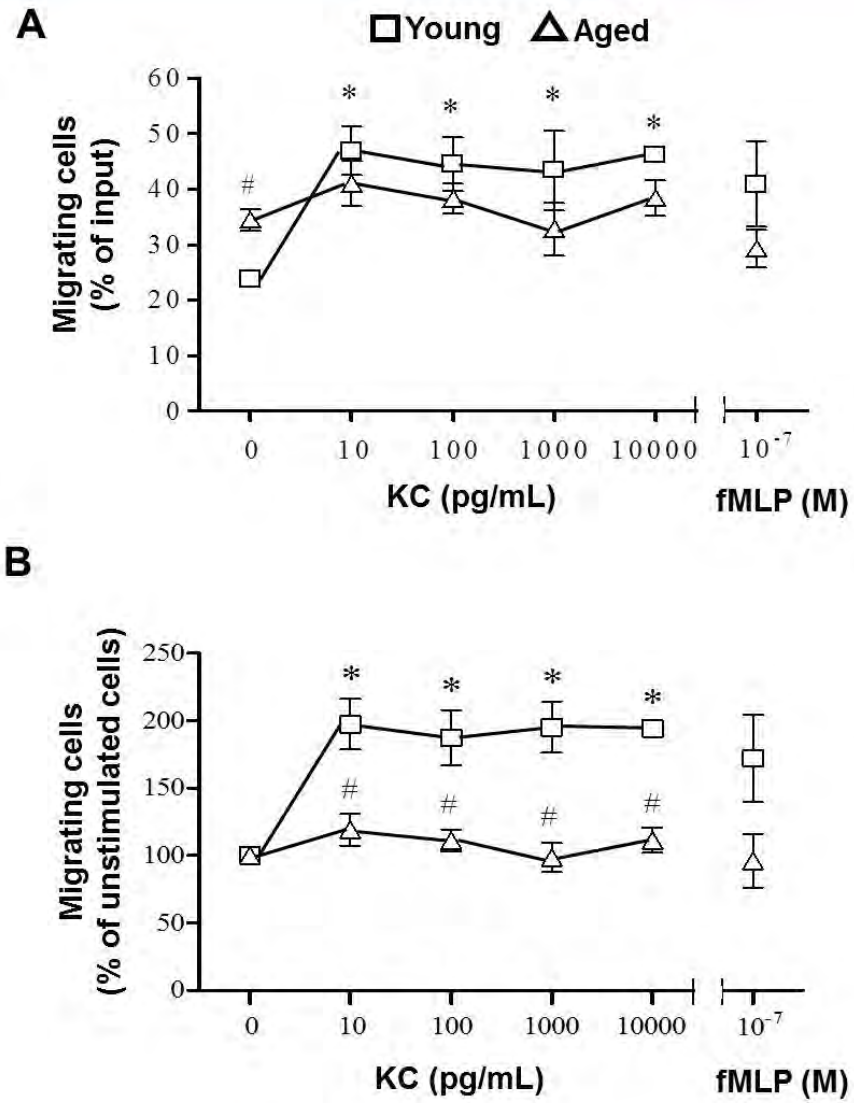

Figure 3. *In vitro* neutrophil chemotaxis assays. Peripheral blood neutrophils isolated from young (squares) and aged (triangles) mice were tagged with CellTracker Green and incubated with varying concentrations of KC in a transwell plate for 1 hour at 37°C. fMLP was used as a positive control. Migrating cells are expressed as (A) % of input (fluorescence of migrated cells/fluorescence of input x 100) or (B) % of unstimulated cells (% input / % input of unstimulated cells x 100). Data are represented as mean ± SEM. N = 3-6 mice per group; *p<0.05 compared to young unstimulated control; #p<0.05 compared to young at the same chemokine dose by Student's t-test.

Table 2. Peripheral blood neutrophil adhesion molecule expression after burn [a]

	CD62L		CD11b	
	Sham	**Burn**	**Sham**	**Burn**
Young	702 ± 44	501± 12 *	3404 ± 232	3328 ± 206
Aged	670 ± 56	525 ± 13 *	3427 ± 259	3511 ± 398

[a] CD62L and CD11b expression on peripheral blood neutrophils ($F4/80^-$ $Gr\text{-}1^+$ cells) from young and aged mice at 24 hours after sham or burn injury was determined by flow cytometry. Data are shown as mean fluorescent intensity ± SEM. N = 6-13 mice per group; *$p<0.05$ verse young sham by one-way ANOVA. Other data are not significant.

CD62L and CD11b expression on peripheral blood neutrophils

Activation of CXCR2 helps facilitate upregulation of selectins and integrins that mediate loose and firm endothelial adherence and transmigration. Elevation of neutrophil selectins or integrins may translate into increased neutrophil adherence to the vasculature, while the observed downregulation of CXCR2 and lack of neutrophil chemotaxis may prevent transmigration in response to apical KC levels. Thus, we sought to examine neutrophil levels of CD62L and CD11b expression by flow cytometry (Table II). Despite the heightened pulmonary neutrophil congestion and impaired directional motility observed in aged mice, no difference in the frequency of CD62L and CD11b positive neutrophils was seen (data not shown). While the MFI of CD62L was reduced in both young and aged mice subjected to burn injury compared to young sham, no significant difference was noted in CD62L and CD11b expression in aged, burn-injured mice as compared young, burn-injured mice. These data suggest that alterations CD62L and CD11b expression does not contribute to the exacerbated pulmonary inflammatory response or impaired chemotaxis found with advanced age.

Pulmonary vascular ICAM-1 expression in response to burn injury

While no differences in CD62L and CD11b were detected, others have shown that endothelial ICAM-1 is important in neutrophil recruitment to the lungs after injury [12, 39-42]. To further investigate this in our model, lung sections were stained for ICAM-1, expressed by both pulmonary endothelial and epithelial cells, and PECAM-1, a constitutively expressed endothelial marker [43]. Colocalization of ICAM-1 and PECAM-1 was considered to be endothelial ICAM-1 expression (Figure 4A). ICAM-1 expression on the pulmonary vasculature in aged, burn-injured mice was increased greater than 3-fold compared to sham controls ($p<0.05$), while no differences were detected in the lungs of young, burn injured mice (Figure 4B). This suggests that pulmonary vasculature in aged mice may more readily bind activated neutrophils that are passing through the lung, subsequently leading to their retention.

DISCUSSION

In summary, the data presented in this study show for the first time that neutrophils are retained in the lung vasculature and/or interstitial space of aged mice after acute burn injury and do not transmigrate into the alveolar space. This process is related, in part, to an altered pulmonary endothelial adhesion molecule profile and decreased capacity for neutrophil chemotaxis in aged mice compared to young mice at 24 hours after burn. Similar features have been shown in young animals within a few hours of injury; however, this response is transient and return to sham levels by 24 hours [42, 44, 45]. The present findings are compounded by the elevated numbers of neutrophils in the peripheral blood, which may promote enhanced congestion in the absence of adequate clearance in aged mice. The difference in pulmonary endothelial adhesion, as well as neutrophil number and function, in aged mice may contribute to the delayed resolution of the pulmonary inflammatory response as seen in our previous study [31] and might contribute to worse outcomes for this group. Taken together, these observations are important in understanding the pathogenesis of burn injury in aged individuals and suggest that prolonged inflammation may be responsible for the various complications seen in this population.

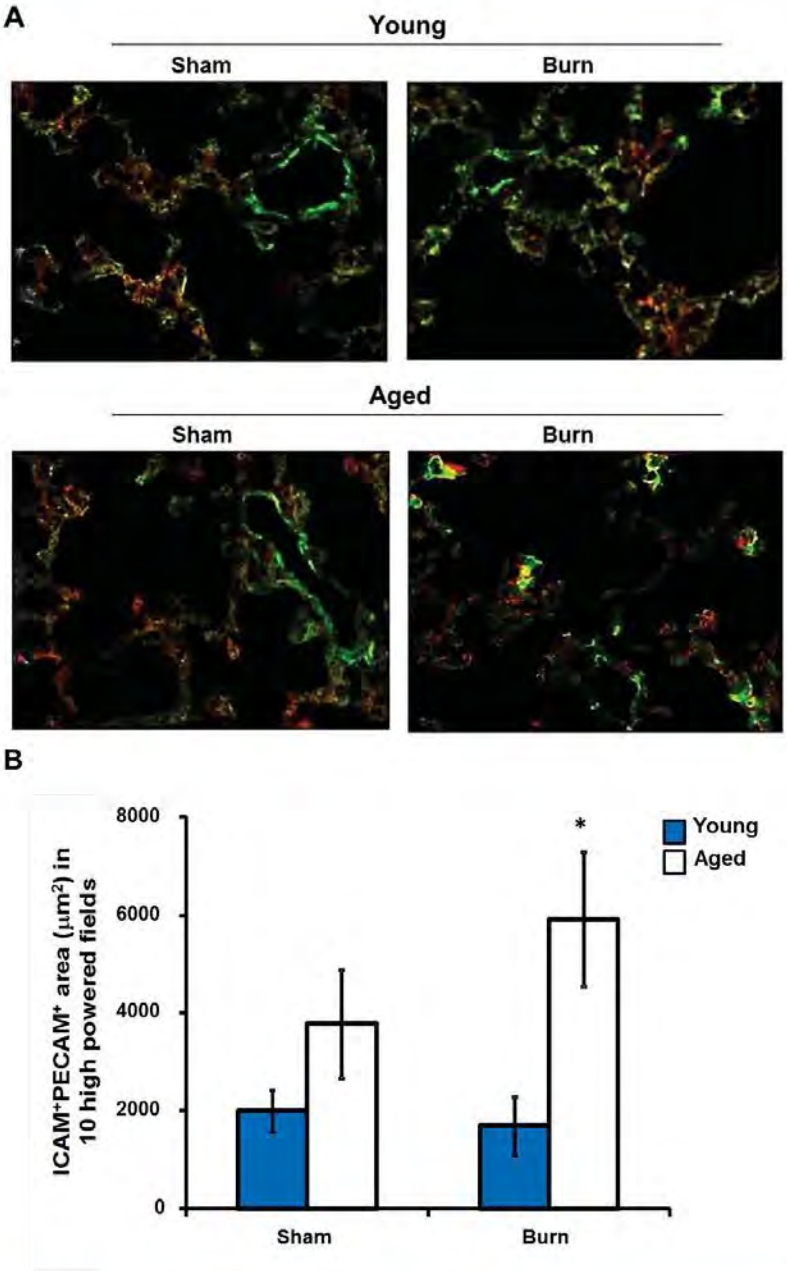

Figure 4. Pulmonary endothelial ICAM-1 expression after burn. Lung sections immunostained with anti-ICAM-1 (red) and anti-PECAM-1 (green) antibodies. (**A**) Representative images are shown from young and aged mice at 24 hours after sham or burn injury at 200x magnification. (**B**) Total ICAM-1$^+$PECAM-1$^+$ area (μm^2) in ten high powered fields (400x). Data are shown as mean $\pm$ SEM. N=8-13 mice per group; *$p<0.05$ compared to young burn by one-way ANOVA.

A prominent theory known as "inflamm-aging" suggests age-associated problems are related to elevations in circulating pro-inflammatory mediators in the absence of clinically detectable disease [46-48]. These findings have been corroborated by others in BAL from aged humans, in which interleukin (IL)-1β and IL-6 were elevated in the absence of injury or disease [49-51]. Previously, we have established that relative to young, traumatic injury in aged mice exacerbates this basal pro-inflammatory state at early time points [14, 31, 36]. Of note, a study by Luu et al. demonstrated that preincubation of neutrophils with various chemokines inhibits transmigration through an activated endothelial monolayer *in vitro* [21][6], suggesting that a persistent pro-inflammatory stimulus can lead to neutrophil dysregulation. Moreover, these neutrophils were able to undergo firm adhesion, but were not able to transmigrate across the endothelial layer. Interestingly, this finding was related to desensitization of the neutrophil chemotatic receptor CXCR2. Currently, our study found that following burn trauma, neutrophils from aged mice exhibit decreased expression of CXCR2. These findings recapitulate observations from human studies in which CXCR2 was downregulated in trauma patients [52, 53]. This reduction may be due to the elevated levels of circulating cytokines and chemokines [31], either through receptor desensitization or receptor ligation and uptake [38, 54, 55]. Regardless, once these neutrophils reach the pulmonary circulation, diminished CXCR2 activity may impair their ability to respond to apical chemokines to mediate transmigration through the endothelium [21, 29].

One interesting finding from this study is that peripheral blood neutrophils from aged mice have an increased ability for random migration, or chemokinesis, compared to those from young mice, as shown by others [48, 56]. Our results indicate that neutrophils from aged mice have a heightened basal level of random migration; however, their ability to respond directionally to a specific stimulus, such as KC, is impaired. While there is some contention in the literature, this idea of heightened basal neutrophil activation in aged mice parallels observations in human studies [57-59]. Advanced age has been associated with increased intracellular Ca^{2+} and G-protein coupled receptor kinase activation [58, 60, 61], both of which modulate neutrophil chemotatic pathways [38, 62-64][7-9]. Similar to the current study, others have shown that neutrophils from aged mice are incapable of generating a peak response once they are exposed to a secondary stimulus, such as direct KC stimulation, live bacteria, or burn injury [58, 61]. Further studies characterizing the direct role of CXCR2 in our model will potentially elucidate this mechanism.

In addition to the chemotatic role of CXCR2, reports indicate that the ability of neutrophils to signal through CXCR2 is important for adherence to and migration through endothelial layers [19, 21, 65, 66]. Activation of CXCR2 leads to upregulation and clustering of selectins, like CD62L, and integrins such as Mac-1 (CD11b/CD18), lymphocyte-function associated antigen-1 (LFA-1: CD11a/CD18) and very late antigen-4 (VLA-4: CD49a/CD29) [24-26]. Specifically, signaling via CXCR2 and other chemokine pathways induce a conformational change in CD11b, allowing it to form a stronger interaction with ICAM-1 on endothelium and undergo adhesion [42, 66, 67]. Due to the differences in CXCR2 expression, we hypothesized that age may be associated with differences in adhesion molecules CD62L and CD11b. However, levels of CD62L and CD11b were found to be comparable between young and aged mice, pre- and post-burn. Additional studies are required to examine the role of CD11b clustering in this model, as well as other neutrophil integrins such as CD11a/CD18 and CD49a/CD29, to determine whether aging alters neutrophil adhesion molecules that promote firm adhesion to the pulmonary endothelium.

Another component critical to neutrophil adhesion and subsequent transmigration is the expression of various adhesion molecules by the pulmonary vasculature. Here, we demonstrate that aged mice have increased expression of pulmonary endothelial ICAM-1 and this correlates with elevated neutrophil numbers within lung tissue. Previous studies in combined trauma models have shown that loss of ICAM-1 decreases neutrophil recruitment into the lung [68], suggesting that targeting ICAM-1 may ameliorate the neutrophil congestion seen in our study. Moreover, elevated levels of pro-inflammatory mediators can activate endothelial cells, resulting in upregulation of ICAM-1 and other adhesion molecules and a pro-adherent endothelium [17, 69, 70]. We have previously demonstrated that following burn, aged animals have elevated circulating levels of pro-inflammatory cytokines and chemokines which likely promotes upregulation of ICAM-1. Moreover, human studies have revealed the cross-linking of ICAM-1 stimulates production of IL-8, a human neutrophil chemokine [71]. This upregulation in IL-8 may be to further enhance neutrophil chemoattraction, but may also aid in the neutrophil transmigratory response to apical chemokines. Additional investigation into vascular adhesion molecule-1 (VCAM-1) signaling, as well as other adhesion molecules such as VCAM-1, will further elucidate the impact of age and trauma on lung endothelium.

Taken together, our data suggest that a combined defect in CXCR2 signaling and elevated ICAM-1 promotes neutrophil congestion in the lungs of aged

mice following burn trauma. The elevated ICAM-1 may immobilize these neutrophils within the vasculature, but their inability to adequately respond to local chemokines may prevent their eventual diapedesis and clearance. We propose that the inflammatory environment of the aged animal primes neutrophils and renders them incapable of responding appropriately to an inflammatory insult, such as burn injury. Moreover, the exacerbated pro-inflammatory environment in aged mice following burn trauma may promote an adherent phenotype within the pulmonary vasculature, further compounding the observed neutrophil defects. Understanding the mechanism involved in this process is important for identifying potential targets of therapy with the ultimate goal of improving outcomes for aged patients sustaining a burn or other traumatic injury.

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